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UTILITY DIVISIONAL PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.: 4147-7-1DIV4

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Title: "NOVEL APOPTOSIS-MODULATING PROTEINS, DNA ENCODING THE PROTEINS AND METHODS OF USE THEREOF"

Group Art Unit:

Examiner:

Commissioner of Patents
Box Patent Application
Washington, DC 20231

This is a Divisional application of pending prior application Serial No. 08/320,157, filed February 14, 1998. The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied, is considered to be part of the disclosure of the accompanying application and is hereby incorporated by reference.

Enclosed for filing with the above-identified utility patent application, please find the following:

1. [X] Copy of Oath/Declaration from the above-referenced pending prior application (37 CFR 1.63(d))
2. [X] Return Postcard (MPEP 503) *(should be specifically itemized)*
3. [X] A check in the amount of $690.00 is enclosed.
4. [X] Other: Copy of the Request for Extension of Time of 3 Months submitted in pending prior application Serial No. 08/320,157.

FEE CALCULATION:

Cancel in this application original Claims 1-31 and 39-58 of the prior application before calculating the filing fee.

	(COL. 1) NO. FILED			(COL. 2*) NO. EXTRA	SMALL ENTITY RATE	SMALL ENTITY FEE		LARGE ENTITY RATE	LARGE ENTITY FEE
BASIC FEE:						$345.00	OR		$690.00
TOTAL CLAIMS:	7	-	20	0	X $9 =		OR	X $18 =	$0.00
INDEP. CLAIMS:	2	-	3	0	X $39 =		OR	X $78 =	$0.00
____ MULTIPLE DEPENDENT CLAIMS					+ $130 =		OR	+$260 =	$0.00
*IF THE DIFFERENCE IN COL. 2 IS LESS THAN ZERO, ENTER "0" IN COL. 2.					TOTAL:				$690.00

OTHER INFORMATION:

1. [X] The Commissioner is hereby authorized to debit any underpayments or credit any overpayment to Deposit Account No. 19-1970.

2. [X] The Commissioner is hereby authorized to charge all required fees for extensions of time under §1.17 to Deposit Account No. 19-1970.

3. [ ] Foreign Priority benefits are claimed under 35 USC §119 of _____ Patent Application Serial No. _____ filed _____.

4. [X] The Power of Attorney appears in the original papers of the prior pending application.

5. [X] The prior application is assigned to LXR Biotechnology, Inc..

6. [ ] This application is being filed by less than all of the inventors named in the pending prior application. The Commissioner is requested to delete the name(s) of the following person(s) from the prior application who are not inventors being claimed in the application: .

7. [X] This is a sequence case. The computer readable form in this application is identical with that filed in the prior application, Serial No. 08/320,157, filed February 14, 1998. In accordance with 37 CFR 1.821(3), please use the computer readable form filed in that application as the computer readable form for the instant application. It is understood that the Patent and Trademark Office will make the necessary change in application number and filing date for the computer readable form that will be used in the instant application. A paper copy of the Sequence Listing is included in the present filing of the instant application. Attorney for Applicants hereby asserts pursuant to 37 CFR § 1.821(f) that the content of the paper copy submitted herewith is identical to the computer readable form submitted in Application No. 08/320,157, filed February 14, 1998.

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Respectfully submitted,

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Theresa A. Brown

Date: 7 August 2000

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re the Application of:

KIEFER et al.

Serial No.: 08/320,157

Filed: February 14, 1998

Atty. File No.: 4147-7-1

For: "NOVEL APOPTOSIS-MODULATING PROTEINS, DNA ENCODING THE PROTEINS AND METHODS OF USE THEREOF"

Group Art Unit: 1642

Examiner: EYLER, Y.

REQUEST FOR EXTENSION OF TIME

CERTIFICATE OF MAILING

I HEREBY CERTIFY THAT THIS CORRESPONDENCE IS BEING DEPOSITED WITH THE UNITED STATES POSTAL SERVICE AS FIRST CLASS MAIL IN AN ENVELOPE ADDRESSED TO ASSISTANT COMMISSIONER FOR PATENTS, WASHINGTON, D.C., 20231 ON AUGUST 7, 2000.

SHERIDAN ROSS P.C.

BY: _____

Box AF
Commissioner of Patents
Washington, D.C. 20231

Dear Sir:

Applicants, through their attorneys, respectfully petition for an extension of time under 37 CFR § 1.136(a) of three (3) months to respond to the Office Action mailed on February 7, 2000, with respect to the above-identified application, thereby extending the period for response from May 7, 2000 to August 7, 2000.

Enclosed is a check in the amount of $435.00 as payment of the extension fee. Please credit any overpayment or debit any underpayment to Deposit Account No. 19-1970.

Respectfully submitted,

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NOVEL APOPTOSIS-MODULATING PROTEINS, DNA ENCODING THE PROTEINS AND METHODS OF USE THEREOF

This is a continuation-in-part of United States patent application Serial No. 08/160,067 filed November 30, 1993.

Field of the Invention

This invention relates to novel proteins with apoptosis-modulating activity, recombinant DNA encoding the proteins, compositions containing the proteins and methods of use thereof.

Background of the Invention

Apoptosis is a normal physiologic process that leads to individual cell death. This process of programmed cell death is involved in a variety of normal and pathogenic biological events and can be induced by a number of unrelated stimuli. Changes in the biological regulation of apoptosis also occur during aging and are responsible for many of the conditions and diseases related to aging. Recent studies of apoptosis have implied that a common metabolic pathway leading to cell death may be initiated by a wide variety of signals, including hormones, serum growth factor deprivation, chemotherapeutic agents, ionizing radiation and infection by human immunodeficiency virus (HIV). Wyllie (1980) Nature, 284:555-556; Kanter et al. (1984) Biochem. Biophys. Res. Commun. 118:392-399; Duke and Cohen (1986) Lymphokine Res. 5:289-299; Tomei et al. (1988) Biochem. Biophys. Res. Commun. 155:324-331; Kruman et al. (1991)

J. Cell. Physiol. 148:267-273; Ameisen and Capron (1991) Immunology Today 12:102; and Sheppard and Ascher (1992) J. AIDS 5:143. Agents that modulate the biological control of apoptosis thus have therapeutic utility in a wide variety of conditions.

Apoptotic cell death is characterized by cellular shrinkage, chromatin condensation, cytoplasmic blebbing, increased membrane permeability and interchromosomal DNA cleavage. Kerr et al. (1992) FASEB J. 6:2450; and Cohen and Duke (1992) Ann. Rev. Immunol. 10:267. The blebs, small, membrane-encapsulated spheres that pinch off of the surface of apoptotic cells, may continue to produce superoxide radicals which damage surrounding cell tissue and may be involved in inflammatory processes.

Bcl-2 was discovered at the common chromosomal translocation site t(14:18) in follicular lymphomas and results in aberrant over-expression of bcl-2. Tsujimoto et al. (1984) Science 226:1097-1099; and Cleary et al. (1986) Cell 47:19-28. The normal function of bcl-2 is the prevention of apoptosis; unregulated expression of bcl-2 in B cells is thought to lead to increased numbers of proliferating B cells which may be a critical factor in the development of lymphoma. McDonnell and Korsmeyer (1991) Nature 349:254-256; and, for review see, Edgington (1993) Bio/Tech. 11:787-792. Bcl-2 is also capable of blocking of γ irradiation-induced cell death. Sentman et al. (1991) Cell 67:879-888; and Strassen (1991) Cell 67:889-899. It is now known that bcl-2 inhibits most types of apoptotic cell death and is thought to function by regulating an antioxidant pathway at sites of free radical generation. Hockenbery et al. (1993) Cell 75:241-251.

While apoptosis is a normal cellular event, it can also be induced by pathological conditions and a

variety of injuries. Apoptosis is involved in a wide variety of conditions including but not limited to, cardiovascular disease, cancer regression, immunoregulation, viral diseases, anemia, neurological disorders, gastrointestinal disorders, including but not limited to, diarrhea and dysentery, diabetes, hair loss, rejection of organ transplants, prostate hypertrophy, obesity, ocular disorders, stress and aging.

Bcl-2 belongs to a family of proteins some of which have been cloned and sequenced. Williams and Smith (1993) Cell 74:777-779. All references cited herein, both supra and infra, are hereby incorporated by reference herein.

Summary of the Invention

Substantially purified DNA encoding novel bcl-2 homologs, termed cdn-1, cdn-2 and cdn-3, as well as recombinant cells and transgenic animals expressing the cdn-1 and cdn-2 genes are provided. The substantially purified CDN-1 and CDN-2 proteins and compositions thereof are also provided. Diagnostic and therapeutic methods utilizing the DNA and proteins are also provided. Methods of screening for pharmaceutical agents that stimulate, as well as pharmaceutical agents that inhibit cdn-1 and cdn-2 activity levels are also provided.

Brief description of the Drawings

Figure 1 depicts the PCR primers used to isolate the cdn-1 probes.

Figure 2 depicts the cdn-1 clones obtained by the methods described in Example 1.

Figure 3 depicts the nucleotide sequence of cdn-1.

Figure 4 depicts the results of a Northern blot analysis of multiple tissues with probes specific for both bcl-2 and cdn-1.

Figure 5 shows the sequence of the cdn-2 cDNA and flanking sequences and the corresponding predicted amino acid sequence of the cdn-2 protein.

Figure 6 shows a comparison of N-terminal amino acid sequences of cdn-1, cdn-2 and known bcl-2 family members.

Figure 7 shows the nucleotide sequence of cdn-3.

Figure 8 shows the anti-apoptotic effects of cdn-1 and some of its derivatives in serum-deprivation induced apoptosis of WIL-2 cells.

Figure 9 shows anti-apoptotic effects of cdn-1 and some of its derivatives in FAS-induced apoptosis of WIL-2 cells.

Figure 10 shows modulation of apoptosis by cdn-1 and cdn-2 in FL5.12 cells.

Figure 11 depicts the cdn-1 derivative proteins Δ1, Δ2 and Δ3. The N-terminal residues are indicated by the arrows. The remainder of the derivative proteins is the same as full-length cdn-1.

Detailed Description of the Invention

The present invention encompasses substantially purified nucleotide sequences encoding the novel bcl-2 homologs, cdn-1 and cdn-2; and the proteins encoded thereby; compositions comprising cdn-1 and cdn-2 genes and proteins and methods of use of thereof. Note that in copending United States patent application Serial No. 08/160,067, cdn-1 was termed cdi-1; although the name has been changed, the nucleotide sequence remains identical. The invention further includes recombinant cells and transgenic animals expressing the cloned cdn-1 or cdn-2

genes. The nucleotide and predicted amino acid residue sequences of cdn-1 are shown in Figure 3; and those of cdn-2 are shown in Figure 5. It has now been found that the proteins encoded by the cdn genes are capable of modulating apoptosis. In a lymphoblastoid cell line, cdn-1 was shown to decrease Fas-mediated apoptosis. In a mouse progenitor B cell line, FL5.12, cdn-2 and a derivative of cdn-1 decrease IL-3-induced apoptosis whereas cdn-1 slightly increased apoptosis. Thus, depending on the cell type, the derivative of cdn and the method of induction of apoptosis, apoptosis can be modulated in a highly specific manner by controlling the concentration of cdns.

As used herein, "cdns" or "cdn" refers to the nucleic acid molecules described herein (cdn-1, cdn-2, cdn-3 and derivatives thereof), "the CDNs" or "CDN" refers to the proteins encoded thereby (CDN-1, CDN-2, CDN-3 and derivatives thereof). The present invention encompasses cdn-1 and cdn-2 nucleotide sequences. The cdn nucleotides include, but are not limited to, the cDNA, genome-derived DNA and synthetic or semi-synthetic DNA or RNA. The nucleotide sequence of the cdn-1 cDNA with the location of restriction endonuclease sites is shown in Figure 2. As described in the examples herein, cdn-1 mRNA has been detected in a variety of human organs and tissues by Northern blot analysis. These organs include liver; heart; skeletal muscle; lung; kidney; and pancreas as shown in Figure 3.

Similarly, cdn-2, cdn cDNA, genomic DNA and synthetic or semi-synthetic DNAs and RNAs are additional embodiments of the present invention. The nucleotide sequence of cdn-2 cDNA, along with the predicted amino acid sequence of cdn-2 protein and the locations of restriction endonuclease recognition sites, is given in Figure 5. The examples presented herein indicate that

cdn-1 is on human chromosome 6 and that cdn-2 is on human chromosome 20. There is also a member of the family cdn-3 which is on human chromosome 11. Fluorescence in situ hybridization (FISH) indicated an approximate location of cdn-1 to be at 6p21-23. Within this region resides the gene for spinocerebellar ataxia type 1. Interestingly, apoptosis has been proposed recently to be involved in the related genetic disorder ataxia telangiectasia. Taken together with the chromosomal localization and the expression of cdn-1 in brain tissue, this suggests the possibility that cdn-1/cdn-2 might represent the SCA1 gene locus. It is possible that cdn-2 and cdn-3 are pseudogenes. While these may not be expressed endogenously, they are capable of expression from a recombinant vector providing the appropriate promoter sequences. Thus, both cdn-2 and cdn-3 genes are encompassed by the present invention as are recombinant constructs thereof and proteins encoded thereby.

Derivatives of the genes and proteins include any portion of the protein, or gene encoding the protein, which retains apoptosis modulating activity. Figure 10 depicts three such derivatives of cdn-1 which have been shown to retain apoptosis-modulating activity. These derivatives, cdn1-Δ1, cdn1-Δ2 and cdn1-Δ3, are encompassed by the present invention.

The invention includes modifications to cdn DNA sequences such as deletions, substitutions and additions particularly in the non-coding regions of genomic DNA. Such changes are useful to facilitate cloning and modify gene expression.

Various substitutions can be made within the coding region that either do not alter the amino acid residues encoded or result in conservatively substituted amino acid residues. Nucleotide substitutions that do not alter the amino acid residues encoded are useful for

optimizing gene expression in different systems. Suitable substitutions are known to those of skill in the art and are made, for instance, to reflect preferred codon usage in the particular expression systems.

The invention encompasses functionally equivalent variants and derivatives of cdns which may enhance, decrease or not significantly affect the properties of CDNs. For instance, changes in the DNA sequence that do not change the encoded amino acid sequence, as well as those that result in conservative substitutions of amino acid residues, one or a few amino acid deletions or additions, and substitution of amino acid residues by amino acid analogs are those which will not significantly affect its properties.

Amino acid residues which can be conservatively substituted for one another include but are not limited to: glycine/alanine; valine/isoleucine/leucine; asparagine/glutamine; aspartic acid/glutamic acid; serine/threonine; lysine/arginine; and phenylalanine/tyrosine. Any conservative amino acid substitution which does not significantly affect the properties of CDNs is encompassed by the present invention.

Techniques for nucleic acid manipulation useful for the practice of the present invention are described in a variety of references, including but not limited to, Molecular Cloning: A Laboratory Manual, 2nd ed., Vol. 1-3, eds. Sambrook et al. Cold Spring Harbor Laboratory Press (1989); and Current Protocols in Molecular Biology, eds. Ausubel et al., Greene Publishing and Wiley-Interscience: New York (1987) and periodic updates.

The invention further embodies a variety of DNA vectors having cloned therein the cdn nucleotide sequences encoding. Suitable vectors include any known

in the art including, but not limited to, those for use in bacterial, mammalian, yeast and insect expression systems. Specific vectors are known in the art and need not be described in detail herein.

The vectors may also provide inducible promoters for expression of the cdns. Inducible promoters are those which do not allow constitutive expression of the gene but rather, permit expression only under certain circumstances. Such promoters may be induced by a variety of stimuli including, but not limited to, exposure of a cell containing the vector to a ligand, metal ion, other chemical or change in temperature.

These promoters may also be cell-specific, that is, inducible only in a particular cell type and often only during a specific period of time. The promoter may further be cell cycle specific, that is, induced or inducible only during a particular stage in the cell cycle. The promoter may be both cell type specific and cell cycle specific. Any inducible promoter known in the art is suitable for use in the present invention.

The invention further includes a variety of expression systems transfected with the vectors. Suitable expression systems include but are not limited to bacterial, mammalian, yeast and insect. Specific expression systems and the use thereof are known in the art and are not described in detail herein.

The invention encompasses *ex vivo* transfection with cdns, in which cells removed from animals including man are transfected with vectors encoding CDNs and reintroduced into animals. Suitable transfected cells include individual cells or cells contained within whole tissues. In addition, *ex vivo* transfection can include the transfection of cells derived from an animal other than the animal or human subject into which the cells are

ultimately introduced. Such grafts include, but are not limited to, allografts, xenografts, and fetal tissue transplantation.

Essentially any cell or tissue type can be treated in this manner. Suitable cells include, but are not limited to, cardiomyocytes and lymphocytes. For instance, lymphocytes, removed, transfected with the recombinant DNA and reintroduced into an HIV-positive patient may increase the half-life of the reintroduced T cells.

As an example, in treatment of HIV-infected patients by the above-described method, the white blood cells are removed from the patient and sorted to yield the $CD4^+$ cells. The $CD4^+$ cells are then transfected with a vector encoding CDNs and reintroduced into the patient. Alternatively, the unsorted lymphocytes can be transfected with a recombinant vector having at least one cdn under the control of a cell-specific promoter such that only $CD4^+$ cells express the cdn genes. In this case, an ideal promoter would be the CD4 promoter; however, any suitable $CD4^+$ T cell-specific promoter can be used.

Further, the invention encompasses cells transfected *in vivo* by the vectors. Suitable methods of *in vivo* transfection are known in the art and include, but are not limited to, that described by Zhu et al. (1993) Science 261:209-211. *In vivo* transfection by cdns may be particularly useful as a prophylactic treatment for patients suffering from atherosclerosis. Elevated modulation of the levels of CDN could serve as a prophylaxis for the apoptosis-associated reperfusion damage that results from cerebral and myocardial infarctions. In these patients with a high risk of stroke and heart attack, the apoptosis and reperfusion

damage associated with arterial obstruction could be prevented or at least mitigated.

Infarctions are caused by a sudden insufficiency of arterial or venous blood supply due to emboli, thrombi, or pressure that produces a macroscopic area of necrosis; the heart, brain, spleen, kidney, intestine, lung and testes are likely to be affected. Apoptosis occurs to tissues surrounding the infarct upon reperfusion of blood to the area; thus, modulation of CDN levels, achieved by a biological modifier-induced change in endogenous production or by *in vivo* transfection, could be effective at reducing the severity of damage caused by heart attacks and stroke.

Transgenic animals containing the recombinant DNA vectors are also encompassed by the invention. Methods of making transgenic animals are known in the art and need not be described in detail herein. For a review of methods used to make transgenic animals, see, e.g. PCT publication no. WO 93/04169. Preferably, such animals express recombinant cdns under control of a cell-specific and, even more preferably, a cell cycle specific promoter.

In another embodiment, diagnostic methods are provided to detect the expression of cdns either at the protein level or the mRNA level. Any antibody that specifically recognizes CDNs is suitable for use in CDN diagnostics. Abnormal levels of CDNs are likely to be found in the tissues of patients with diseases associated with inappropriate apoptosis; diagnostic methods are therefore useful for detecting and monitoring biological conditions associated with such apoptosis defects. Detection methods are also useful for monitoring the success of CDN-related therapies.

Purification or isolation of CDNs expressed either by the recombinant DNA or from biological sources

such as tissues can be accomplished by any method known in the art. Protein purification methods are known in the art. Generally, substantially purified proteins are those which are free of other, contaminating cellular substances, particularly proteins. Preferably, the purified CDNs are more than eighty percent pure and most preferably more than ninety-five percent pure. For clinical use as described below, the CDNs are preferably highly purified, at least about ninety-nine percent pure, and free of pyrogens and other contaminants.

Suitable methods of protein purification are known in the art and include, but are not limited to, affinity chromatography, immunoaffinity chromatography, size exclusion chromatography, HPLC and FPLC. Any purification scheme that does not result in substantial degradation of the protein is suitable for use in the present invention.

The invention also includes the substantially purified CDNs having the amino acid residue sequences depicted in Figures 3 and 5, respectively. The invention encompasses functionally equivalent variants of CDNs which do not significantly affect their properties and variants which retain the same overall amino acid sequence but which have enhanced or decreased activity. For instance, conservative substitutions of amino acid residues, one or a few amino acid deletions or additions, and substitution of amino acid residues by amino acid analogs are within the scope of the invention.

Amino acid residues which can be conservatively substituted for one another include but are not limited to: glycine/alanine; valine/isoleucine/leucine; asparagine/glutamine; aspartic acid/glutamic acid; serine/threonine; lysine/arginine; and phenylalanine/tyrosine. Any conservative amino acid substitution which does not significantly affect the

properties of CDNs is encompassed by the present invention.

Suitable antibodies are generated by using the CDNs as an antigen or, preferably, peptides encompassing the CDN regions that lack substantial homology to the other gene products of the bcl family. Methods of detecting proteins using antibodies and of generating antibodies using proteins or synthetic peptides are known in the art and are not be described in detail herein.

CDN protein expression can also be monitored by measuring the level of cdn mRNA. Any method for detecting specific mRNA species is suitable for use in this method. This is easily accomplished using the polymerase chain reaction (PCR). Preferably, the primers chosen for PCR correspond to the regions of the cdn genes which lack substantial homology to other members of the bcl gene family. Alternatively, Northern blots can be utilized to detect cdn mRNA by using probes specific to cdns. Methods of utilizing PCR and Northern blots are known in the art and are not described in detail herein.

Methods of treatment with cdns also include modulating cellular expression of cdns by increasing or decreasing levels of cdn mRNA or protein. Suitable methods of increasing cellular expression of cdn include, but are not limited to, increasing endogenous expression and transfecting the cells with vectors encoding cdns. Cellular transfection is discussed above and is known in the art. Suitable indications for increasing endogenous levels of cdn include, but are not limited to, malignancies and cardiac-specific over-expression. Cardiac specific over-expression is particularly suitable for use in indications including, but not limited to, patients susceptible to heart disease and in advance of cardiotoxic therapies including, but not limited to,

chemotherapies such as adriamycin, so as to offer cardioprotection.

In addition, increasing endogenous expression of cdns can be accomplished by exposing the cells to biological modifiers that directly or indirectly increase levels of CDNs either by increasing expression or by decreasing degradation of cdn mRNA. Suitable biological modifiers include, but are not limited to, molecules and other cells. Suitable molecules include, but are not limited to, drugs, cytokines, small molecules, hormones, combinations of interleukins, lectins and other stimulating agents e.g. PMA, LPS, bispecific antibodies and other agents which modify cellular functions or protein expression. Cells are exposed to such biological modifiers at physiologically effective concentrations, and the expression of cdns is measured relative to a control not exposed to the biological modifiers. Those biological modifiers which increase expression of cdns relative to the control are selected for further study.

The invention further encompasses a method of decreasing endogenous levels of cdns. The methods of decreasing endogenous levels of cdns include, but are not limited to, antisense nucleotide therapy and down-regulation of expression by biological modifiers. Antisense therapy is known in the art and its application will be apparent to one of skill in the art.

Screening for therapeutically effective biological modifiers is done by exposing the cells to biological modifiers which may directly or indirectly decrease levels of CDNs either by decreasing expression or by increasing the half-life of cdn mRNA or CDNs. Suitable biological modifiers include, but are not limited to, molecules and other cells. Suitable molecules include, but are not limited to, drugs, cytokines, small molecules, hormones, combinations of

interleukins, lectins and other stimulating agents e.g. PMA, LPS, bispecific antibodies and other agents which modify cellular functions or protein expression. Cells are grown under conditions known to elicit expression of at least one cdn (preferably cdn-1), exposed to such biological modifiers at physiologically effective concentrations, and the expression of cdns is measured relative to a control not exposed to biological modifiers. Those biological modifiers which decrease the expression of cdns relative to a control are selected for further study. Cell viability is also monitored to ensure that decreased cdn expression is not due to cell death.

In determining the ability of biological modifiers to modulate (increase or decrease) cdn expression, the levels of endogenous expression may be measured or the levels of recombinant fusion proteins under control of cdn-specific promoter sequences may be measured. The fusion proteins are encoded by reporter genes.

Reporter genes are known in the art and include, but are not limited to chloramphenicol acetyl transferase (CAT) and β-galactosidase. Expression of cdn-1 and -2 can be monitored as described above either by protein or mRNA levels. Expression of the reporter genes can be monitored by enzymatic assays, or antibody-based assays, like ELISAs and RIAs, also known in the art. Potential pharmaceutical agents can be any therapeutic agent or chemical known to the art, or any uncharacterized compounds derived from natural sources such as fungal broths and plant extracts. Preferably, suitable pharmaceutical agents are those lacking substantial cytotoxicity and carcinogenicity.

Suitable indications for modulating endogenous levels of cdns are any in which cdn-mediated apoptosis is

involved. These include, but are not limited to, various types of malignancies and other disorders resulting in uncontrolled cell growth such as eczema, or deficiencies in normal programmed cell death such as malignancies, including, but not limited to, B cell lymphomas.

The invention also encompasses therapeutic methods and compositions involving treatment of patients with biological modifiers to increase or decreast expression of cdns. Effective concentrations and dosage regimens may be empirically derived. Such derivations are within the skill of those in the art and depend on, for instance, age, weight and gender of the patient and severity of the disease. Alternatively, patients may be directly treated with either native or recombinant CDNs. The CDNs should be substantially pure and free of pyrogens. It is preferred that the recombinant CDNs be produced in a mammalian cell line so as to ensure proper glycosylation. CDNs may also be produced in an insect cell line and will be glycosylated.

For therapeutic compositions, a therapeutically effective amount of substantially pure CDN is suspended in a physiologically accepted buffer including, but not limited to, saline and phosphate buffered saline (PBS) and administered to the patient. Preferably administration is intravenous. Other methods of administration include but are not limited to, subcutaneous, intraperitoneal, gastrointestinal and directly to a specific organ, such as intracardiac, for instance, to treat cell death related to myocardial infarction.

Suitable buffers and methods of administration are known in the art. The effective concentration of a CDN will need to be determined empirically and will depend on the type and severity of the disease, disease

progression and health of the patient. Such determinations are within the skill of one in the art.

Bcl-2 is thought to function in an antioxidant pathway. Veis et al. (1993) Cell 75:229-240. Therefore, therapy involving CDNs is suitable for use in conditions in which superoxide is involved. Administration of CDNs results in an increased extracellular concentration of CDNs, which is thought to provide a method of directly inhibiting superoxide accumulation that may be produced by the blebs associated with apoptosis. The therapeutic method thus includes, but is not limited to, inhibiting superoxide mediated cell injury.

Suitable indications for therapeutic use of CDNs are those involving free radical mediated cell death and include, but are not limited to, conditions previously thought to be treatable by superoxide dismutase. Such indications include but are not limited to HIV infection, autoimmune diseases, cardiomyopathies, neuronal disorders, hepatitis and other liver diseases, osteoporosis, and shock syndromes, including, but not limited to, septicemia.

Hybridization of cloned cdn DNA to messenger mRNA from various regions of the brain indicated high levels of expression of cdn-1 in each of the regions studied (Figure 8). Therefore, neurological disorders are another area in which therapeutic applications of CDNs may be indicated.

The following examples are provided to illustrate but not limit the present invention. Unless otherwise specified, all cloning techniques were essentially as described by Sambrook et al. (1989) and all reagents were used according to the manufacturer's instructions.

Example 1

Identification and Cloning of cdn-1 cDNA

An amino acid sequence comparison of the six known bcl-2 family members (Figure 6) revealed two regions with considerable sequence identity, namely amino acids 144-150 and 191-199. In an attempt to identify new bcl-2 family members, degenerate PCR primers based on sequences in these regions were designed (Figure 1) and PCR was performed using human heart cDNA and human B lymphoblastoid cell line (WIL-2) cDNA. PCR was performed using the Hot Start/Ampliwax technique (Perkin Elmer Cetus). The final concentration of the PCR primers and the template cDNA were 4 μM and 0.1-0.2 ng/ml, respectively. The conditions for cDNA synthesis were identical to those for first strand cDNA synthesis of the cDNA library as described below. PCR was performed in a Perkin Elmer Cetus DNA Thermal Cycler according to the method described by Kiefer et al. (1991) Biochem. Biophys. Res. Commun. 176:219-225, except that the annealing and extension temperatures during the first 10 cycles were 36°C. Following PCR, samples were treated with 5 units of DNA polymerase I, Klenow fragment for 30 min at 37°C and then fractionated by electrophoresis on a 7% polyacrylamide, 1 X TBE (Tris/borate/EDTA) gel. DNA migrating between 170-210 base pars was excised from the gel, passively eluted for 16 hours with gentle shaking in 10 mM Tris-HCl pH 7.5, 1 mM EDTA (TE), purified by passage over an Elutip-D column (Schleicher and Schuell), ligated to the pCR-Script vector (Stratagene) and transformed into *Escherichia coli* strain XL1-Blue MRF (Stratagene). Plasmid DNA from transformants (white colonies) containing both the heart and WIL-2 PCR products was isolated using the Magic Miniprep DNA Purification System (Promega), and the DNA inserts were sequenced by the dideoxy chain termination method

according to Sanger et al. (1977) Proc. Natl. Acad. Sci. USA 74:5463-5467 (USB, Sequenase version 2.0). DNA sequence analysis of the eleven heart PCR products revealed two sequences identical to bcl-x (Boise et al. (1993) Cell 74:597-608) and ten other sequences unrelated to the bcl-2 family.

DNA sequence analyses of the eleven WIL-2 PCR products yielded one bcl-x sequence, five sequences identical to another bcl-2 family member, bax (Oldvai et al. (1993) Cell 74:609-619), four unrelated sequences and one novel bcl-2 related sequence, termed cdn-1. The unique cdn-1 amino acid sequence encoded by the PCR product is shown in Figure 6 from amino acid 151-190 (top row).

To isolate the cdn-1 cDNA, a human heart cDNA library (Clontech) and a WIL-2 cDNA library, constructed as described by Zapf et al. (1990) J. Biol. Chem. 265:14892-14898 were screened using the cdn-1 PCR DNA insert as a probe. The DNA was ^{32}P-labeled according to the method described by Feinberg and Vogelstein (1984) Anal. Biochem. 137:266-267 and used to screen 150,000 recombinant clones from both libraries according to the method described by Kiefer et al. (1991). Eight positive clones from the WIL-2 cDNA library and two positive clones from the heart cDNA library were identified. Four clones from the WIL-2 cDNA library and two from the heart cDNA library were further purified and plasmid DNA containing the cDNA inserts was excised from the λZAPII vector (Stratagene) (Figure 2). The two longest clones, W7 (2.1 kb) and W5 (2.0 kb) were sequenced and shown to contain the cdn-1 probe sequence, thus confirming their authenticity. The heart cDNAs also encoded cdn-1.

The W7 DNA sequence along with the deduced amino acid residue sequence is shown in Figure 2. The deduced amino acid sequence of cdn-1 was also aligned for

maximum sequence identity with the other bcl-2 family members and is shown in Figure 6. As can be seen, there is considerable sequence identity between cdn-1 and other family members between amino acids 100 and 200. Beyond this central region, sequence conservation falls off sharply. Like bcl-2, cdn-1 appears to be an intracellular protein in that it does not contain a either a hydrophobic signal peptide or N-linked glycosylation sites. Cdn-1 does contain a hydrophobic C-terminus that is also observed with all bcl-2 family members except LMW5-HL, suggesting its site of anti-apoptotic activity, like that of bcl-2, is localized to a membrane bound organelle such as the mitochondrial membrane, the endoplasmic reticulum or the nuclear membrane. Hockenbery et al. (1990); Chen-Levy et al. (1989) Mol. Cell. Biol. 9:701-710; Jacobsen et al. (1993) Nature 361:365-369; and Monighan et al. (1992) J. Histochem. Cytochem. 40:1819-1825.

Example 2

Northern Blot Analysis of cDNA Clones

Northern blot analysis was performed according to the method described by Lehrach et al. (1977) Biochem. 16:4743-4651 and Thomas (1980) Proc. Natl. Acad. Sci. USA 77:5201-5205. In addition, a human multiple tissue Northern blot was purchased from Clontech. The coding regions of bcl-2 and cdn-1 cDNAs were labeled by the random priming method described by Feinberg and Vogelstein (1984) Anal. Biochem. 137:266-267. Hybridization and washing conditions were performed according to the methods described by Kiefer et al. (1991).

The results, presented in Figure 4 indicate that cdn-1 is expressed in all organs tested (heart, brain, placenta, lung, liver, skeletal muscle, kidney and

pancreas) whereas bcl-2 is not expressed or expressed at only low levels in heart, brain, lung, and liver. Thus, cdn-1 appears to be more widely expressed throughout human organs than bcl-2 and may be more important in regulating apoptosis in these tissues.

Example 3

Expression of Recombinant cdn-1

In order to express recombinant cdn-1 in the baculovirus system, the cdn-1 cDNA generated in Example 1 was used to generate a novel cdn-1 vector, by a PCR methodology as described in Example 1, using primers from the 3' and 5' flanking regions of the gene which contain restriction sites to facilitate cloning. The plasmids were sequenced by the dideoxy terminator method (Sanger *et al.*, 1977) using sequencing kits (USB, Sequenase version 2.0) and internal primers. This was to confirm that no mutations resulted from PCR.

A clone was used to generate recombinant viruses by *in vivo* homologous recombination between the overlapping sequences of the plasmid and AcNPV wild type baculovirus. After 48 hours post-transfection in insect *Spodoptera frugiperda* clone 9 (SF9) cells, the recombinant viruses were collected, identified by PCR and further purified. Standard procedures for selection, screening and propagation of recombinant baculovirus were performed (Invitrogen). The molecular mass, on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), of the protein produced in the baculovirus system was compared with the predicted molecular mass of cdn-1 according to the amino-acid sequence.

In addition, similar clones can be expressed preferably in a yeast intracellular expression system by any method known in the art, including the method

described by Barr et al. (1992) Transgenesis ed. JAH Murray, (Wiley and Sons) pp. 55-79.

Example 4

Expression of cdn-1 in Mammalian Systems

The cdn-1 coding sequence was excised from a plasmid generated in Example 1, and introduced into plasmids pCEP7, pREP7 and pcDNA3 (Invitrogen) at compatible restriction enzyme sites. pCEP7 was generated by removing the RSV 3'-LTR of pREP7 with XbaI/Asp718, and substituting the CMV promoter from pCEP4 (Invitrogen). 25 μg of each cdn-1-containing plasmid was electroporated into the B lymphoblastoid cell line WIL-2, and stable hygromycin resistant transformants or G418 resistant transformants (pcDNA3 constructs, Fig. 8) expressing cdn-1 were selected.

The coding region of cdns can also ligated into expression vectors capable of stably integrating into other cell types including but not limited to cardiomyocytes, neural cell lines such as GTI-7 and TNF sensitive cells such as the human colon adenocarcinoma cell line HT29 so as to provide a variety of assay systems to monitor the regulation of apoptosis by cdn-1.

Example 5

Effect of the Anti-Apoptotic Activity of cdn-1 and its Derivatives in the Wild Type B Lymphoblastoid Cell Line WIL2-729 HF2 and the Transformed Cell Expressing Excess cdn-1

$2x10^5$ WIL-2, and WIL-2 cells transformed with a vector encoding cdn-1 as described in Example 4 are grown in RPMI supplemented with 10% fetal bovine serum (FBS) for the anti-fas experiment or 0.1% FBS for serum deprivation experiments. In the case of the anti-fas experiment, after washing with fresh medium, the cells

were suspended in RPMI supplemented with 10% FBS, exposed to anti-fas antibodies and the kinetics of cell death in response to an apoptosis inducing agent were analyzed by flow cytometry with FACScan. In the case of the serum deprivation experiment, the WIL-2 cells were resuspended in RPMI supplemented with 0.1% FBS and apoptosis was monitored according to the method described by Henderson et al. (1993) Proc. Natl. Acad. Sci. USA 90:8479-8483. Other methods of inducing apoptosis include, but are not limited to, oxygen deprivation in primary cardiac myocytes, NGF withdrawal, glutathione depletion in the neural cell line GTI-7 or TNF addition to the HT29 cell line. Apoptosis was assessed by measuring cell shrinkage and permeability to propidium iodide (PI) during their death. In addition, any other method of assessing apoptotic cell death may be used.

Figure 8 shows the anti-apoptotic response of various WIL-2 transformants to anti-Fas treatment. Figure 9 shows the anti-apoptotic response of various WIL-2 transformants to serum deprivation. In Figure 8, duplicate wells containing $3x10^5$ cells were incubated with 50 ng/ml of the cytocidal anti-Fas antibody for 24 hours. Cell death was then analyzed by flow cytometry with FACScan. The proteins expressed from each construct are shown beneath the columns. Since many of the constructs are truncation or deletion variants, the exact amino acids expressed are also indicated. As can be seen, all of the transformants had some protective effect when compared to the control transformant containing the pREP7 vector alone. The most apoptosis-resistant transformant was the cdn-1Δ2 expressing cell line, in which over 90% of the cells survived anti-fas treatment. Significant protection was also observed in transformants expressing full length cdn-1 (1-211) and cdn-1Δ1, followed by bcl-2Δ and bcl-2 expressing cell lines.

Cdn-1Δ1 and cdn-1Δ2 are lacking the N-terminal 59 and 70 amino acids of the full length cdn-1 molecule, respectively. The observation that cdn-1Δ2 is more effective at blocking apoptosis than full length cdn-1 suggests that smaller, truncated cdn-1 molecules may be potent therapeutics.

Example 6

Determination of other cdn genes and

Cloning of the cdn-2 Gene

Southern blot analyses of human genome DNA and a panel of human/rodent somatic cell DNAs indicated that there were at least 3 cdn related genes and that they resided in chromosomes 6, 11 and 20. PCR/sequence analysis of the three hybrid DNAs showed that cdn-1 was on chromosome 6 and that two closely related sequences were on chromosome 20 (designated cdn-2) and chromosome 11 (designated cdn-3). We have cloned the cdn-2 and cdn-3 genes and sequenced them. Interestingly, both cdn-2 and cdn-3 do not contain introns and have all of the features of processed genes that have returned to the genome. cdn-3 has a nucleotide deletion, causing a frame shift and early termination and thus is probably a pseudogene. Both, however, have promoter elements upstream of the repeats CCAAT, TATAAA boxes but are probably not transcribed. (Northern blot analysis with cdn-2 and cdn-3 specified probes.)

900,000 clones from a human placenta genomic library in the cosmid vector pWE15 (Stratagene, La Jolla, CA) were screened with a 950 bp BglII- HindIII cDNA probe containing the entire coding region of Cdn-1. The probe was ^{32}P-labeled according to the method of Feinberg and Vogelstein (1984) Anal. Biochem. 137:266-267. The library was processed and screened under high stringency hybridization and washing conditions as described by

Sambrook et al. (1989) Molecular Cloning, 2nd edition, Cold Spring Harbor Laboratory Press. Ten double positive clones were further purified by replating and screening as above. Plasmid DNA was purified using the Wizard Maxiprep DNA Purification System as described by the supplier (Promega Corp., Madison, WI) and analyzed by EcoRI restriction enzyme mapping and Southern blotting. The probe used for Southern blotting and hybridization conditions was the same as above.

The cosmid clones fell into two groups as judged by EcoRI restriction analysis and Southern blotting. Cosmid clones (cos) 1-4 and 7 displayed one distinct pattern of EcoRI generated DNA fragments and contained a single 6.5 kb hybridizing EcoRI DNA fragment. Cos2 and Cos9 fell into the second group that was characterized by a 5.5 kb hybridizing EcoRI DNA fragment. The 6.5 kb DNA fragment from cos2 and the 5.5 kb DNA fragment from cos9 were subcloned into pBluescript SK- (Stratagene, La Jolla, CA) using standard molecular biological techniques (Sambrook et al. as above). Plasmid DNA was isolated and the DNA inserts from two subclones, A4 (from cos2) and C5 (from cos9) were mapped with BamHI, HindIII and EcoRI and analyzed by Southern blotting as described above. Smaller restriction fragments from both clones were subcloned into M13 sequencing vectors and the DNA sequence was determined.

The sequence of A4 contains an open reading frame that displays 97% amino acid sequence identity with cdn-1. (Figure 5) The high degree of sequence identity of this gene with cdn-1 indicates that it is a new cdn-1 related gene and therefore will be called cdn-2. A sequence comparison of the encoded cdn-2 protein and the other members of the bcl-2 family is shown in Figure 5. Cdn-2 contains the conserved regions, BH1 and BH2, that are hallmarks of the bcl-2 family, and displays a lower

overall sequence identity (~20-30%) to other members, which is also characteristic of the bcl-2 family. cdn-3 has a frame shift and therefore does not contain the structural features of cdn-1, cdn-2 or other bcl-2 family members.

Example 7

Chromosomal Localization of the cdn-1 and cdn-2 Genes

Southern blot analysis of a panel of human/rodent somatic cell hybrid DNAs (Panel #2 DNA from the NIGMS, Camden, NJ) and fluorescent in situ hybridization (FISH) of metaphase chromosomes were used to map the cdn genes to human chromosomes. For Southern blotting, 5μg of hybrid panel DNA was digested with EcoRI or BamHI/HindIII, fractionated on 0.8% or 1% agarose gels, transferred to nitrocellulose and hybridized with the cdn-1 probe. Hybridization and washing conditions were as described above. For FISH, the cdn-2 subclone, A4, was biotinylated using the Bionick Labeling System (Gibco BRL, Gaithersburg, MD) and hybridized to metaphase chromosomes from normal human fibroblasts according to the method described by Viegas-Pequignot in In Situ Hybridization, A Practical Approach, 1992, ed. D.G. Wilkinson, pp. 137-158, IRL Press, Oxford. Probe detection using FITC-conjugated avidin and biotinylated goat anti-avidin was according to the method described by Pinkel et al. (1988) Proc. Natl. Acad. Sci. USA 85:9138-9142.

Southern blot analysis showed three hybridizing EcoRI bands in the human DNA control that were approximately 12 kb, 11 kb and 5.5 kb in length. Analysis of the somatic cell hybrid DNA indicated that the 12 kb band was in two different samples, NA10629, which contained only human chromosome 6, and NA07299, which contained both human chromosomes 1 and X and,

importantly, a portion of chromosome 6 telomeric to p21. The 11 kb band was in NA13140, which contains human chromosome 20. The 5.5 kb hybridizing band was found only in sample NA10927A, which contained human chromosome 11. PCR/DNA sequencing analysis of these hybrid DNA samples using primers for cdn-1 or cdn-2, showed cdn-1 sequences in NA10629 (the chromosome 6-containing hybrid DNA) and NA07299 (the chromosome 1, X and 6pter >p21-containing hybrid DNA), indicating that the cdn-1 gene resides on chromosome 6, telomeric to p21. cdn-2 sequences were found in NA13140, indicating the cdn-2 gene resides on chromosome 20, and cdn-3 sequences were found in NA10927A, indicating the cdn-3 gene resides on chromosome 11.

Example 8

Modulation of apoptosis by cdn-1 and cdn-2 in FL5.12 cells

FL5.12 is an IL-3-dependent lymphoid progenitor cell line (McKearn et al. (1985) Proc. Natl. Acad. Sci USA 82:7414-7418) that has been shown to undergo apoptosis following withdrawal of IL-3 but is protected from cell death by overexpression of bcl-2. Nunez et al. (1990) J. Immunol. 144:3602-3610; and Hockenbery et al. (1990) Nature 348:334-336. To assess the ability of cdn-1 and cdn-2 to modulate apoptosis, cDNAs encoding cdn-1, cdn-2, two truncated forms of cdn-1 (described below) and bcl-2 were ligated into the mammalian expression vector, pcDNA3 (Invitrogen, San Diego, CA) and stably introduced into the mouse progenitor B lymphocyte cell line FL5.12 by electroporation and selection in media containing the antibiotic G418. Assays were then performed on bulk transformants as described below.

The effects of the overexpressed genes on FL5.12 cell viability were examined at various times

following withdrawal of IL-3 and are shown in Figure 10. Cell viability was assessed by propidium iodide (PI) exclusion on a flow cytometer (Becton Dickinson FACScan). Bcl-2 expression protected the cells significantly from cell death while cdn-1 appeared to enhance cell death when compared to the vector control. Cdn-2 expression conferred a low level of protection from cell death at earlier times but was insignificant at later time points. Interestingly, cdn-1Δ2 gave a moderate level of protection against cell death. Cdn-1-112, a molecule that contains the N-terminal 112 amino acids of cdn-1, also appeared to partially protect the FL5.12 cells although at lower levels than Bcl-2.

As shown in Example 7, expression of cdn-1 and cdn-1Δ2 in WIL2 cells resulted in increased cell survival in response to anti-Fas-mediated apoptosis and serum withdrawal. Taken together, these data suggest that the various cdn molecules are capable of modulating apoptosis in a positive or negative manner, depending on the cell type and apoptotic stimuli. Thus, they are effective in preventing cell death such as in the post-ischemic reperfusion tissue damage in the heart or in inducing cell death in cells that have escaped apoptotic control, as is the case in various cancers.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to those skilled in the art that certain changes and modifications may be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

We claim:

1. A composition comprising a substantially purified nucleotide sequence encoding a cdn.

2. The composition according to claim 1 wherein the nucleotide sequence is derived from genomic DNA.

3. The composition according to claim 1 wherein the cdn is cdn-1.

4. The composition accoding to claim 3 having the nucleotide sequence depicted in Figure 3.

5. The composition according to claim 1 wherein the cdn is cdn-2.

6. The composition according to claim 5 having the nucleotide sequence depicted in Figure 5.

7. A composition comprising a recombinant DNA vector encoding a cdn.

8. The composition according to claim 7 wherein the CDN is CDN-1.

9. The composition according to claim 8 wherein the nucleotide sequence is depicted in Figure 3.

10. The composition according to claim 7 wherein the CDN is CDN-2.

11. The composition according to claim 10 wherein the nucleotide sequence is depicted in Figure 5.

12. The recombinant DNA vector according to claim 7 wherein expression of the sequence encoding the cdn under control of an inducible promoter.

13. A composition comprising a cell transfected with a recombinant DNA vector encoding a cdn.

14. The composition according to claim 13 wherein the CDN-1.

15. The composition according to claim 14 wherein the nucleotide sequence is depicted in Figure 3.

16. The composition according to claim 13 wherein the CDN is CDN-2.

17. The composition according to claim 16 wherein the nucleotide sequence is depicted in Figure 5.

18. A transgenic animal comprising a recombinant DNA vector encoding a CDN.

19. The transgenic animal according to claim 18 wherein the CDN is CDN-1.

20. The transgenic animal according to claim 19 wherein the cdn nucleotide sequence is depicted in Figure 3.

21. The transgenic animl according to claim 18 wherein the CDN is CDN-2.

22. The transgenic animal according to claim 21 wherein the cdn nucleotide sequence is depicted in Figure 5.

23. A composition comprising a substantially purified CDN protein.

24. The composition according to claim 23 wherein the CDN is CDN-1.

25. The composition according to claim 24 wherein the nucleotide sequence is depicted in Figure 3.

26. The composition according to claim 23 wherein the CDN is CDN-2.

27. The composition according to claim 26 wherein the nucleotide sequence is depicted in Figure 5.

28. The composition according to claim 23 wherein the proteins are expressed by recombinant DNA.

29. The composition according to claim 23 wherein the proteins are native proteins.

30. A composition comprising the proteins according to claim 23 and a pharmaceutically acceptable buffer.

31. The composition according to claim 30 wherein the proteins are present in therapeutically effective amounts.

32. A composition comprising a monoclonal or polyclonal antibody which recognizes a CDN but is substantially unreactive with other members of the bcl family.

33. A method of detecting the presence of a CDN protein in a biological sample comprising the steps of:

a) obtaining a cell sample;

b) lysing or permeabilizing the cells to antibodies;

c) adding anti-cdns-specific antibodies to the cell sample;

d) maintaining the cell sample under conditions that allow the antibodies to complex with the cdn; and

e) detecting the antibody-cdn complexes formed.

34. The method according to claim 33 wherein the CDN is CDN-1.

35. The method according to claim 34 wherein the nucleotide sequence is depicted in Figure 3.

36. The method according to claim 33 wherein the CDN is CDN-2.

37. The method according to claim 36 wherein the nucleotide sequence is depicted in Figure 5.

38. The method according to claim 32 wherein the cell sample comprises T cells.

39. A method for detecting the expression of a cdn gene in a biological sample comprising the steps of identifying the presence of RNA encoding the cdn.

40. The method according to claim 39 wherein the method for identifying the cdn-1 or cdn-2 mRNA is Northern blotting.

41. A method identifying cdn mRNA comprising the steps of:

a) obtaining a cell sample;

b) obtaining RNA from the cell sample;

c) performing a polymerase chain reaction on the RNA using primers corresponding to unique regions of the cdn; and

d) detecting the presence of products of the polymerase chain reaction.

42. A method of modulating apoptosis-induced cell death comprising modulating the endogenous levels of a CDN.

43. The method according to claim 40 wherein the CDN is CDN-1.

44. The method according to claim 43 wherein the nucleotide sequence is depicted in Figure 3.

45. The method according to claim 42 wherein the CDN is CDN-2.

46 . The method according to claim 45 wherein the nucleotide sequence is depicted in Figure 5.

47. The method according to claim 41 wherein the CDN is increased by modulating expression of an endogenous cdn gene.

48. The method according to claim 46 wherein the cdn gene expressed is encoded by a recombinant gene.

49. The method according to claim 48 wherein expression of the gene is under the control of an inducible promoter.

50. The method according to claim 49 wherein the cells and transfected *ex vivo* and further comprising the steps of reintroducing the transfected cells into the animal.

51. The method according to claim 50 wherein the cells are T lymphocytes.

52. The method according to claim 49 wherein the recombinant gene is transfected into cells *in vivo*.

53. A method of treating apoptosis in a patient in need thereof comprising administering a therapeutically effective amount of CDN.

54. The method according to claim 53 wherein the CDN is CDN-1.

55. The method according to claim 54 wherein the nucleotide sequence is depicted in Figure 3.

56. The method according to claim 53 wherein the CDN is CDN-2.

57. The method according to claim 56 wherein the nucleotide sequence is depicted in Figure 5.

58. The method according to claim 53 wherein the CDN is administered for any indication for which superoxide dismutase has been indicated.

Abstract

The present invention provides a novel family of apoptosis-modulating proteins. Nucleotide and amino acid residue sequences and methods of use thereof are also provided.

Figure 1

bcl Consensus PCR Primers

```
                                 Ile
EcoRI AspTrpGlyArgValValAla
5- AGATCTGAATTCAACTTGGGGGIC(A)GIA(G)TXGTXGC -3' bclx 1-32


              AspTrpGlyGlyGlnGluAsnAspGlnIleTrp
                          AGGGTIGGIGGXACXAGA(G)ACA(T)(C)TAGGT
5'- AGATCT'AAGCTTGTCCCAICCICCXTGXTCC(T)TGA(G)ATCCA -3' bclX 2-39
```

Figure 2

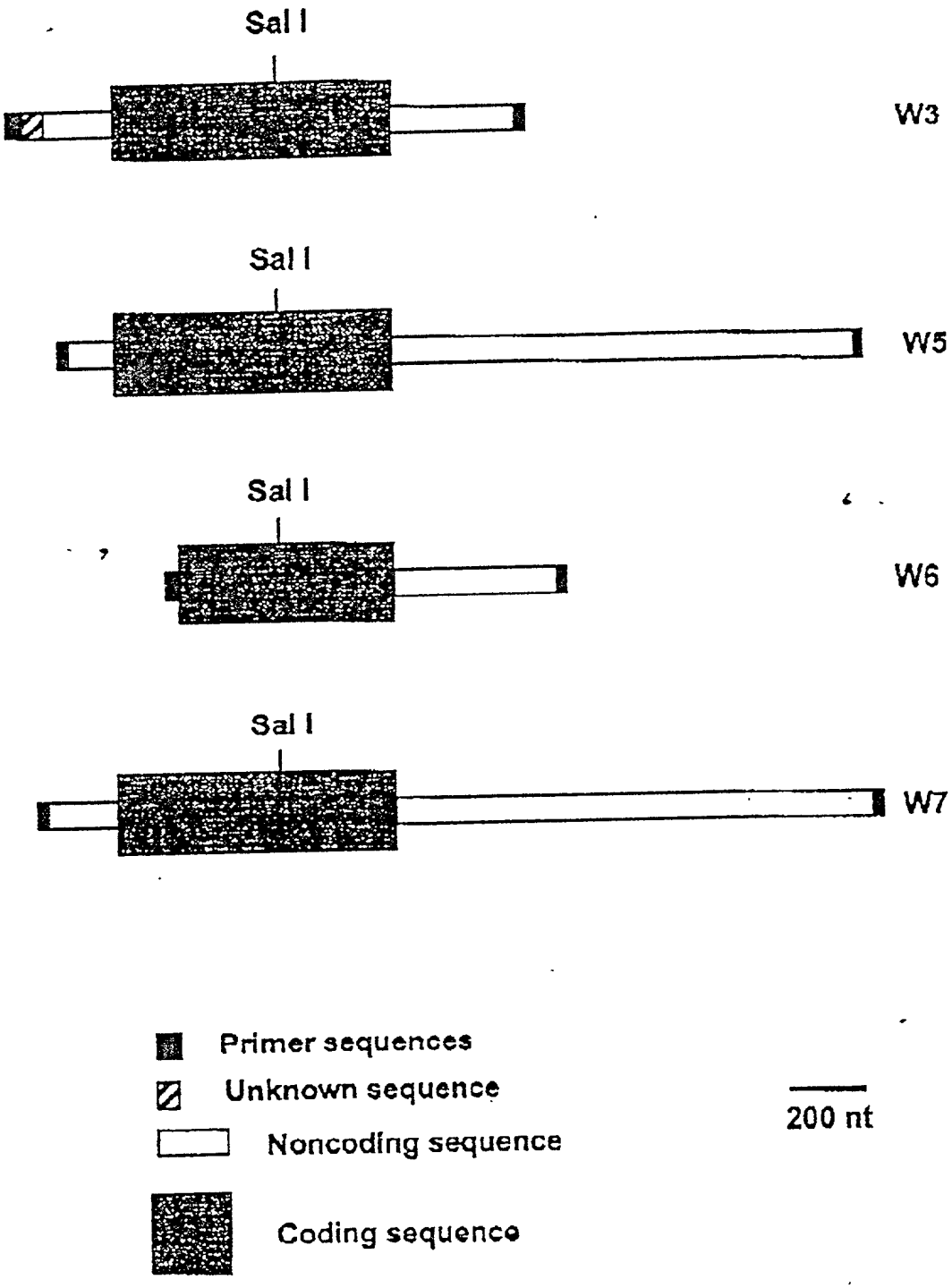
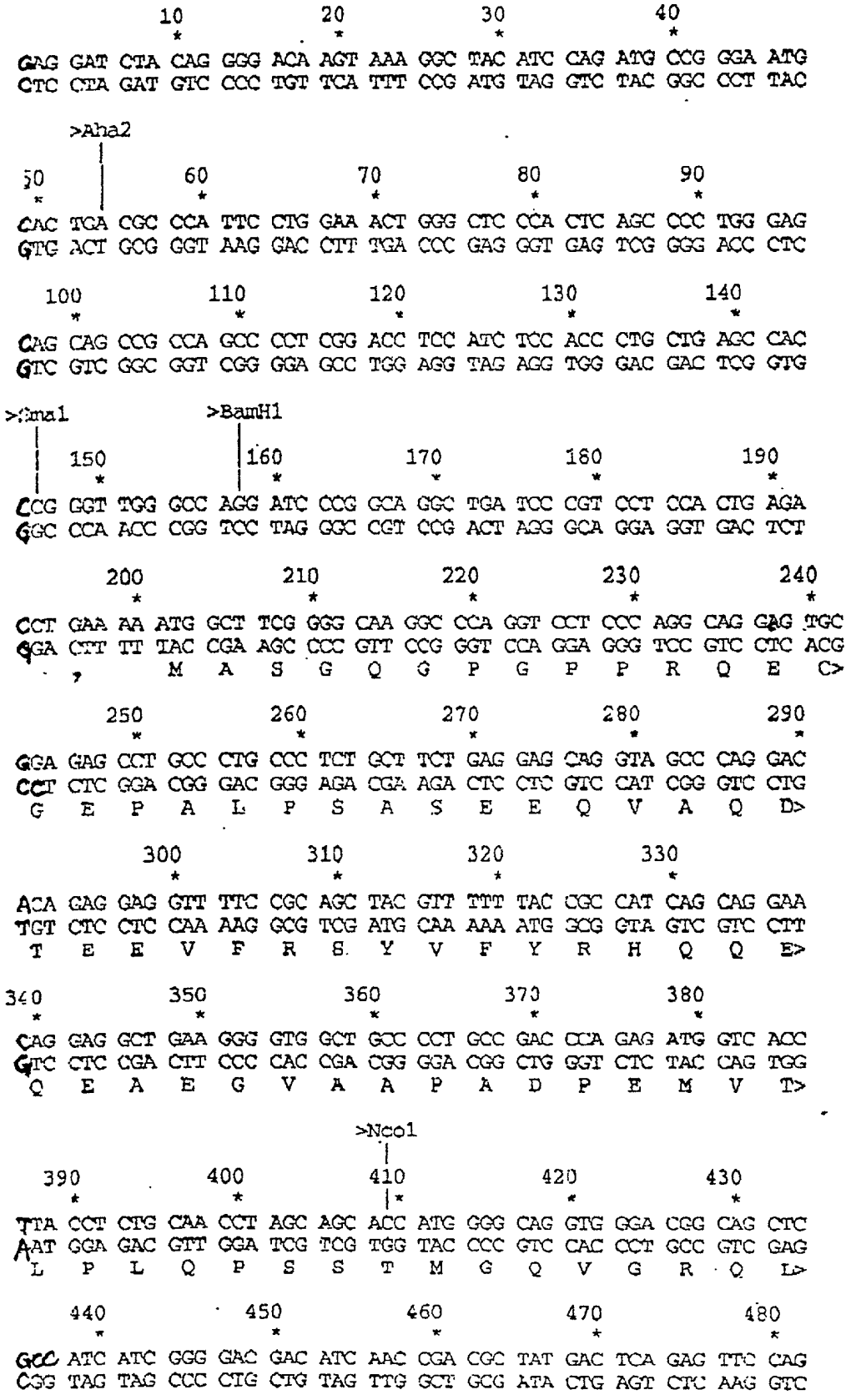

Cdi-1 cDNA clones
Sal I
W3
Sal I
W5
Sal I
W6
Sal I
W7
Primer sequences
Unknown sequence
Noncoding sequence
Coding sequence
200 nt

Figure 3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>Aha2
50 60 70 80 90
CAC TCA CGC CCA TTC CTG GAA ACT GGG CTC CCA CTC AGC CCC TGG GAG
GTG ACT GCG GGT AAG GAC CTT TGA CCC GAG GGT GAG TCG GGG ACC CTC
100 110 120 130 140
CAG CAG CCG CCA GCC CCT CGG ACC TCC ATC TCC ACC CTG CTG AGC CAC
GTC GTC GGC GGT CGG GGA GCC TGG AGG TAG AGG TGG GAC GAC TCG GTG
>Sma1
>BamH1
150 160 170 180 190
CCG GGT TGG GCC AGG ATC CCG GCA GGC TGA TCC CGT CCT CCA CTG AGA
GGC CCA ACC CGG TCC TAG GGC CGT CCG ACT AGG GCA GGA GGT GAC TCT
200 210 220 230 240
CCT GAA AA ATG GCT TCG GGG CAA GGC CCA GGT CCT CCC AGG CAG GAG TGC
GGA CTT TT TAC CGA AGC CCC GTT CCG GGT CCA GGA GGG TCC GTC CTC ACG
, M A S G Q G P G P P R Q E C>
250 260 270 280 290
GGA GAG CCT GCC CTG CCC TCT GCT TCT GAG GAG CAG GTA GCC CAG GAC
CCT CTC GGA CGG GAC GGG AGA CGA AGA CTC CTC GTC CAT CGG GTC CTG
G E P A L P S A S E E Q V A Q D>
300 310 320 330
ACA GAG GAG GTT TTC CGC AGC TAC GTT TTT TAC CGC CAT CAG CAG GAA
TGT CTC CTC CAA AAG GCG TCG ATG CAA AAA ATG GCG GTA GTC GTC CTT
T E E V F R S Y V F Y R H Q Q E>
340 350 360 370 380
CAG GAG GCT GAA GGG GTG GCT GCC CCT GCC GAC CCA GAG ATG GTC ACC
GTC CTC CGA CTT CCC CAC CGA CGG GGA CGG CTG GGT CTC TAC CAG TGG
Q E A E G V A A P A D P E M V T>
>Nco1
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L P L Q P S S T M G Q V G R Q L>
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GCC ATC ATC GGG GAC GAC ATC AAC CGA CGC TAT GAC TCA GAG TTC CAG
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Figure 3 cont.

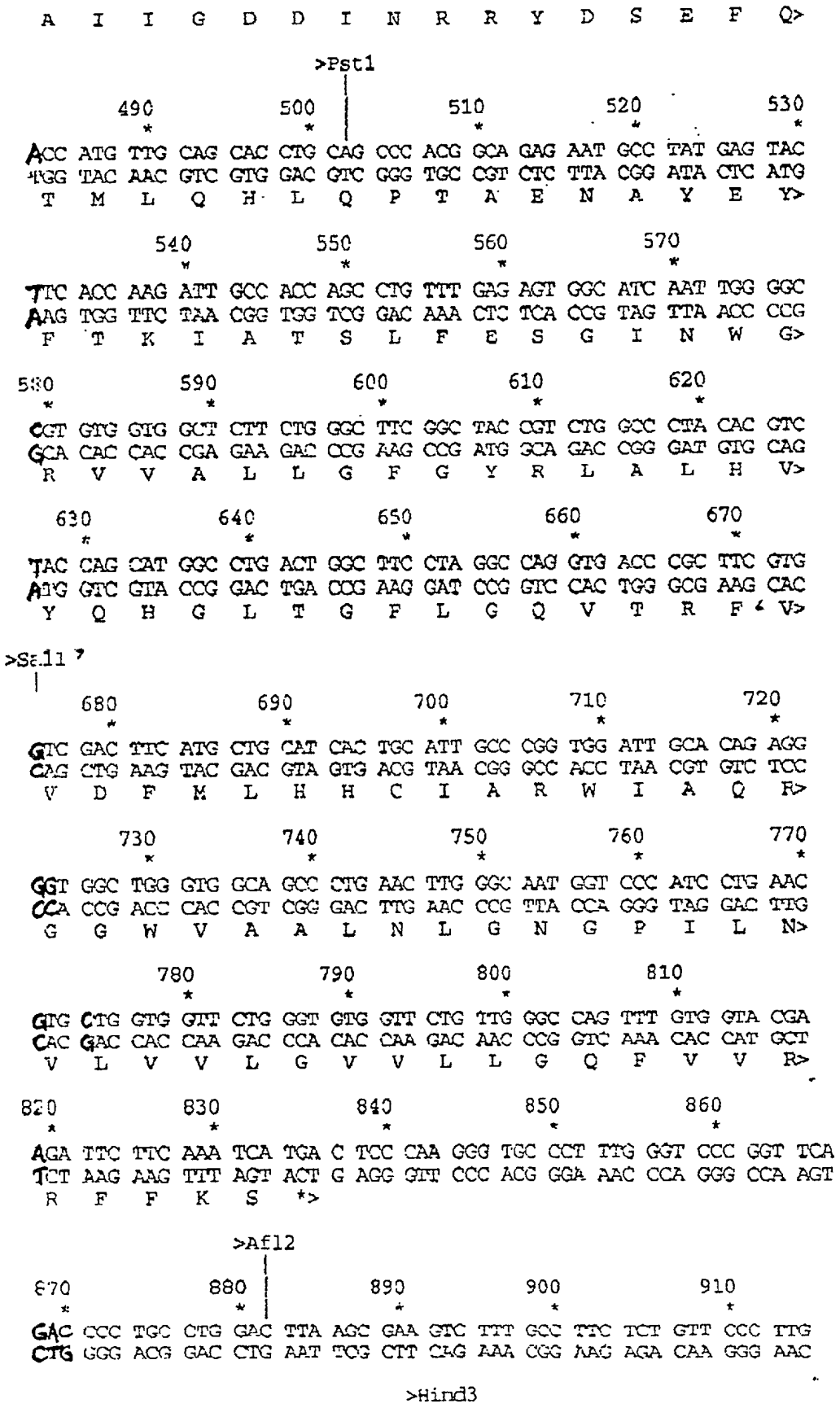


Figure 3 cont.

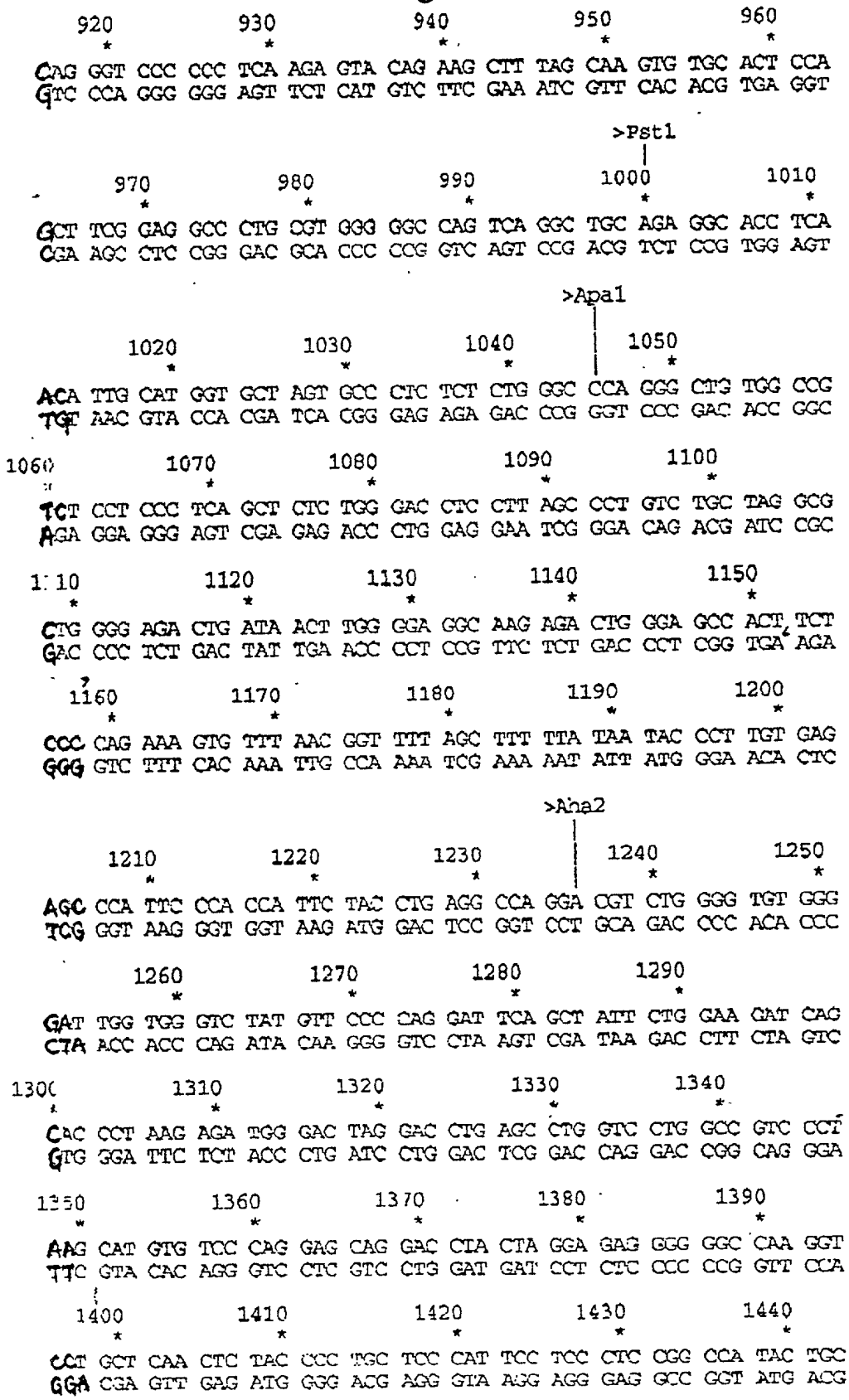


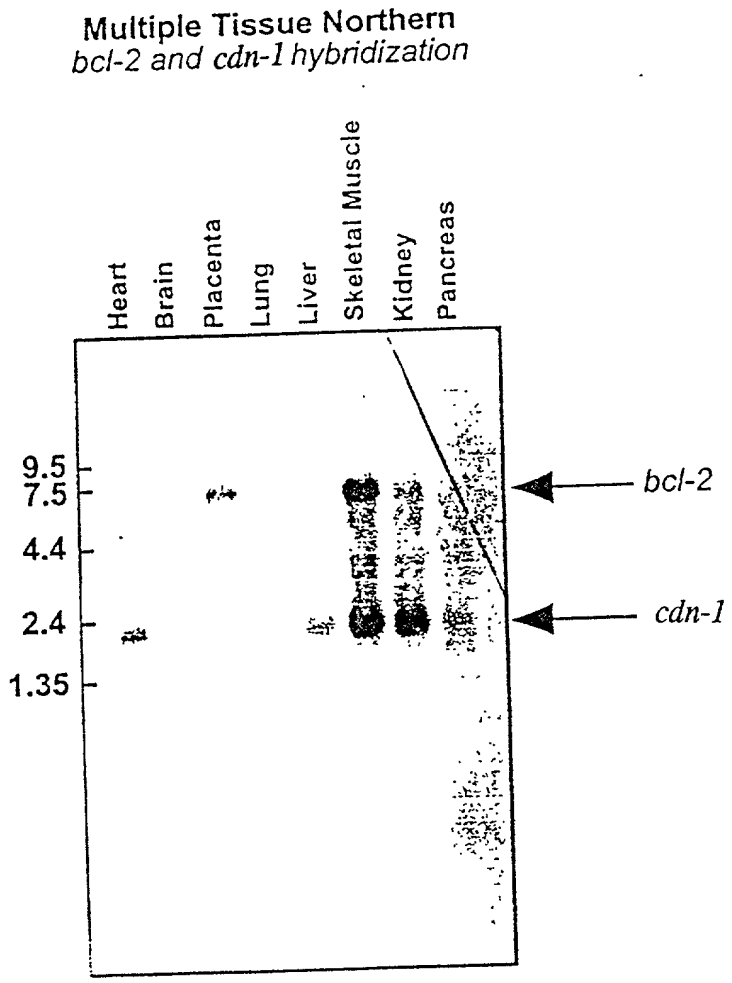
Figure 3 cont.

```
       1450        1460        1470        1480        1490
          *           *           *           *           *
CTT TGC AGT TGG ACT CTC AGG GAT TCT GGG CTT GGG GTG TGG GGT GGG
GAA ACG TCA ACC TGA GAG TCC CTA AGA CCC GAA CCC CAC ACC CCA CCC

          1500        1510        1520        1530
             *           *           *           *
GTG GAG TCG CAG ACC AGA GCT GTC TGA ACT CAC GTG TCA GAA GCC TCC
CAC CTC AGC GTC TGG TCT CGA CAG ACT TGA GTG CAC AGT CTT CGG AGG

1540        1550        1560        1570        1580
   *           *           *           *           *
AAG CCT GCC TCC CAA GGT CCT CTC AGT TCT CTC CCT TCC TCT CTC CTT
TTC GGA CGG AGG GTT CCA GGA GAG TCA AGA GAG GGA AGG AGA GAG GAA

 1590        1600        1610        1620        1630
    *           *           *           *           *
ATA GAC ACT TGC TCC CAA CCC ATT CAC TAC AGG TGA AGG CTC TCA CCC
TAT CTG TGA ACG AGG GTT GGG TAA GTG ATG TCC ACT TCC GAG AGT GGG

    1640        1650        1660        1670        1680
       *           *           *           *           *
ATC CCT GGG GGC CTT GGG TGA GTG GCC TGC TAA GGC TCC TCC TTG CCC
TAG GGA CCC CCG GAA CCC ACT CAC CGG ACG ATT CCG AGG AGG AAC GGG

       1690        1700        1710        1720        1730
          *           *           *           *           *
AGA CTA CAG GGC TTA GGA CTT GGT TTG TTA TAT CAG GGA AAA GGA GTA
TCT GAT GTC CCG AAT CCT GAA CCA AAC AAT ATA GTC CCT TTT CCT CAT

         1740        1750        1760        1770
            *           *           *           *
GGG AGT TCA TCT GGA GGG TTC TAA GTG GGA GAA GGA CTA TCA ACA CCA
CCC TCA AGT AGA CCT CCC AAG ATT CAC CCT CTT CCT GAT AGT TGT GGT

                  >BamH1
                   |
1780        1790   |1800        1810        1820
   *           *   |   *           *           *
CTA GGA ATC CCA GAG GTG GAT CCT CCC TCA TGG CTC TGG CAC AGT GTA
GAT CCT TAG GGT CTC CAC CTA GGA GGG AGT ACC GAG ACC GTG TCA CAT

 1830        1840        1850        1860        1870
    *           *           *           *           *
ATC CAG GGG TGT AGA TGG GGG AAC TGT GAA TAC TTG AAC TCT GTT CCC
TAG GTC CCC ACA TCT ACC CCC TTG ACA CTT ATG AAC TTG AGA CAA GGG

    1880        1890        1900        1910        1920
       *           *           *           *           *
CCA CCC TCC ATG CTC CTC ACC TGT CTA GGT CTC CTC AGG GTG GGG GGT
GGT GGG AGG TAC GAG GAG TGG ACA GAT CCA GAG GAG TCC CAC CCC CCA

       1930        1940        1950        1960        1970
          *           *           *           *           *
GAC AGT GCC TTC TCT ATT GGC ACA GCC TAG GGT CTT GGG GGT CAG GGG
CTG TCA CGG AAG AGA TAA CCG TGT CGG ATC CCA GAA CCC CCA GTC CCC

         1980        1990        2000        2010
            *           *           *           *
GGA GAA GTT CTT GAT TCA GCC AAA TGC AGG GAG GGG AGG CAG ATG GAG
CCT CTT CAA GAA CTA AGT CGG TTT ACG TCC CTC CCC TCC GTC TAC CTC
```

Figure 3 cont.

```
2020      2030      2040       2050      2060
 *         *         *          *         *
CCC ATA GGC CAC CCC CTA TCC TCT GAG TGT TTG GAA ATA AAC TGT GCA
GGG TAT CCG GTG GGG GAT AGG AGA CTC ACA AAC CTT TAT TTG ACA CGT

2070       2080      2090
 *          *         *
ATC CCC TCA AAA AAA AAA CGG AGA TCC
TAG GGG AGT TTT TTT TTT GCC TCT AGG
```

Figure 4



Random primed. Klenow-labeled fragments of bcl-2 and cdn-1 clones were hybridized to a multiple human tissue Northern blot (Clontech 7760-1), at a final concentration of $1x10^6$ cpm/ml for each probe. Blot was washed at high stringency.

Figure 5 cdn-2 gene sequence

```
              10        20        30        40        50        60
               *         *         *         *         *         *
TTT TAA TAT AAA TTA ATG TGC TCT ATT TAT AGA GAC AAT ACA TGA AAT ATA CTT AAT AAA
AAA ATT ATA TTT AAT TAC ACG AGA TAA ATA TCT CTG TTA TGT ACT TTA TAT GAA TTA TTT

              70        80        90       100       110       120
               *         *         *         *         *         *
AAT TCA AAT GTT ATA GAA CTG AAA AAG ATG AAA AGT AAA AAC AAC CTA TTC CCC AGA GGT
TTA AGT TTA CAA TAT CTT GAC TTT TTC TAC TTT TCA TTT TTG TTG GAT AAG GGG TCT CCA

             130       140       150       160       170       180
               *         *         *         *         *         *
AGC CAC TGT CCA TAG TTT CTA TTT TAG ATT CTT TCC TTT ATA CAA GAT TAT TAT AGC TTC
TCG GTG ACA GGT ATC AAA GAT AAA ATC TAA GAA AGG AAA TAT GTT CTA ATA ATA TCG AAG

             190       200       210       220       230       240
               *         *         *         *         *         *
TAT TTT TTG GTG TAT GAA CTG TAG TCC TAG AGG ATT TTA TTA GTT ATG AGT TCT ATA ACT
ATA AAA AAC CAC ATA CTT GAC ATC AGG ATC TCC TAA AAT AAT CAA TAC TCA AGA TAT TGA

             250       260       270       280       290       300
               *         *         *         *         *         *
AAG ATC CAT CAT CTT AGT TGC TAA GAA CGT AGA TAC TGA GAA CAT CAT TTA AAA AAA CAT
TTC TAG GTA GTA GAA TCA ACG ATT CTT GCA TCT ATG ACT CTT GTA GTA AAT TTT TTT GTA

             310       320       330       340       350       360
               *         *         *         *         *         *
TTT TGG CTG GCA CCT CAT GAT CAC TGG AGT CTC GCG GGT CCC TCA GGC TGC ACA GGG ACA
AAA ACC GAC CGT GGA GTA CTA GTG ACC TCA GAG CGC CCA GGG AGT CCG ACG TGT CCC TGT

             370       380       390       400       410       420
               *         *         *         *         *         *
AGT AAA GGC TAC ATC CAG ATG CTG GGA ATG CAC TGA CGC CCA TTC CTG GAA ACT GGG CTC
TCA TTT CCG ATG TAG GTC TAC GAC CCT TAC GTG ACT GCG GGT AAG GAC CTT TGA CCC GAG

             430       440       450       460       470       480
               *         *         *         *         *         *
CCA CTC AGC CCC TGG GAG CAG CAG CCG CCA GCC CCT CGG GAC CTC CAT CTC CAC CCT GCT
GGT GAG TCG GGG ACC CTC GTC GTC GGC GGT CGG GGA GCC CTG GAG GTA GAG GTG GGA CGA

                      >BamHI
             490       500|      510       520       530       540
               *         *|        *         *         *         *
GAG CCA CCC GGG TTG GGC CAG GAT CCC GGC AGG CTG ATC CCG TCC TCC ACT GAG ACC TGA
CTC GGT GGG CCC AAC CCG GTC CTA GGG CCG TCC GAC TAG GGC AGG AGG TGA CTC TGG ACT

             550       560       570       580       590       600
               *         *         *         *         *         *
AAA ATG GCT TCG GGG CAA GGC CCA GGT CCT CCC AGG CAG GAG TGC GGA GAG CCT GCC CTG
TTT TAC CGA AGC CCC GTT CCG GGT CCA GGA GGG TCC GTC CTC ACG CCT CTC GGA CGG GAC
     M   A   S   G   Q   G   P   G   P   P   R   Q   E   C   G   E   P   A   L>

             610       620       630       640       650       660
               *         *         *         *         *         *
CCC TCT GCT TCT GAG GAG CAG GTA GCC CAG GAC ACA GAG GAG GTT TTC CGC AGC TAC GTT
GGG AGA CGA AGA CTC CTC GTC CAT CGG GTC CTG TGT CTC CTC CAA AAG GCG TCG ATG CAA
 P   S   A   S   E   E   Q   V   A   Q   D   T   E   E   V   F   R   S   Y   V>

             670       680       690       700       710       720
               *         *         *         *         *         *
TTT TAC CAC CAT CAG CAG GAA CAG GAG GCT GAA GGG GCG GCT GCC CCT GCC GAC CCA GAG
AAA ATG GTG GTA GTC GTC CTT GTC CTC CGA CTT CCC CGC CGA CGG GGA CGG CTG GGT CTC
 F   Y   H   H   Q   Q   E   Q   E   A   E   G   A   A   A   P   A   D   P   E>
```

Figure 5 cont.

```
                                                    >Nco1
          730           740           750           |           760           770           780
            *             *             *           |             *             *             *
ATG GTC ACC TTA CCT CTG CAA CCT AGC AGC ACC ATG GGG CAG GTG GGA CGG CAG CTC GCC
TAC CAG TGG AAT GGA GAC GTT GGA TCG TCG TGG TAC CCC GTC CAC CCT GCC GTC GAG CGG
 M   V   T   L   P   L   Q   P   S   S   T   M   G   Q   V   G   R   Q   L   A>

          790           800           810           820           830           840
            *             *             *             *             *             *
ATC ATT GGG GAC GAC ATC AAC CGA CGC TAT GAC TCA GAG TTC CAG ACC ATG TTG CAG CAC
TAG TAA CCC CTG CTG TAG TTG GCT GCG ATA CTG AGT CTC AAG GTC TGG TAC AAC GTC GTG
 I   I   G   D   D   I   N   R   R   Y   D   S   E   F   Q   T   M   L   Q   H>

   >Pst1
     |    850           860           870           880           890           900
     |      *             *             *             *             *             *
CTG CAG CCC ACG GCA GAG AAT GCC TAT GAG TAC TTC ACC AAG ATT GCC TCC AGC CTG TTT
GAC GTC GGG TGC CGT CTC TTA CGG ATA CTC ATG AAG TGG TTC TAA CGG AGG TCG GAC AAA
 L   Q   P   T   A   E   N   A   Y   E   Y   F   T   K   I   A   S   S   L   F>

          910           920           930           940           950           960
            *             *             *             *             *             *
GAG AGT GGC ATC AAT TGG GGC CGT GTG GTG GCT CTT CTG GGC TTC AGC TAC CGT CTG GCC
CTC TCA CCG TAG TTA ACC CCG GCA CAC CAC CGA GAA GAC CCG AAG TCG ATG GCA GAC CGG
 E   S   G   I   N   W   G   R   V   V   A   L   L   G   F   S   Y   R   L   A>

          970           980           990          1000          1010          1020
            *             *             *             *             *             *
CTA CAC ATC TAC CAG CGT GGC CTG ACT GGC TTC CTG GGC CAG GTG ACC CGC TTT GTG GTG
GAT GTG TAG ATG GTC GCA CCG GAC TGA CCG AAG GAC CCG GTC CAC TGG GCG AAA CAC CAC
 L   H   I   Y   Q   R   G   L   T   G   F   L   G   Q   V   T   R   F   V   V>

          1030          1040          1050          1060          1070          1080
            *             *             *             *             *             *
GAC TTC ATG CTG CAT CAC TGC ATT GCC CGG TGG ATT GCA CAG AGG GGT GGC TGG GTG GCA
CTG AAG TAC GAC GTA GTG ACG TAA CGG GCC ACC TAA CGT GTC TCC CCA CCG ACC CAC CGT
 D   F   M   L   H   H   C   I   A   R   W   I   A   Q   R   G   G   W   V   A>

          1090          1100          1110          1120          1130          1140
            *             *             *             *             *             *
GCC CTG AAC TTG GGC AAT GGT CCC ATC CTG AAC GTG CTG GTG GTT CTG GGT GTG GTT CTG
CGG GAC TTG AAC CCG TTA CCA GGG TAG GAC TTG CAC GAC CAC CAA GAC CCA CAC CAA GAC
 A   L   N   L   G   N   G   P   I   L   N   V   L   V   V   L   G   V   V   L>

          1150          1160          1170          1180          1190          1200
            *             *             *             *             *             *
TTG GGC CAG TTT GTG GTA CGA AGA TTC TTC AAA TCA TGA CTC CCA AGG GTG CCT TTG GGG
AAC CCG GTC AAA CAC CAT GCT TCT AAG AAG TTT AGT ACT GAG GGT TCC CAC GGA AAC CCC
 L   G   Q   F   V   V   R   R   F   F   K   S   *>

          1210          1220          1230          1240          1250          1260
            *             *             *             *             *             *
TCC CAG TTC AGA CCC CTG CCT GGA CTT AAG CGA AGT CTT TGC CTT CTC TGC TCC TTG CAG
AGG GTC AAG TCT GGG GAC GGA CCT GAA TTC GCT TCA GAA ACG GAA GAG ACG AGG AAC GTC

                              >Hind3
          1270          1280  |
            *             *   |
GGT CCC CCC TCA AGA GTA CAG AAG CTT
CCA GGG GGG AGT TCT CAT GTC TTC GAA
```

Figure 6 Amino acid sequences of cdn-1, cdn-2, and bcl-2 family proteins

```
cdn1                                                                masgqgpgpprqecgepalpsaseeqvaqdteevfrsyvfyrhqqeqeaegvaapadpemvt
cdn2                                                                masgqgpgpprqecgepalpsaseeqvaqdteevfrsyvfyHhqqeqeaegAaapadpemvt
bcl2                                                 mahagrtgyDNREIVMKYIHYKLSQRGYEWdagdvgaappgaapapgifssqpghtphtaasrdpvartsplqtpaapgaa
bax                                                                                 mdgsgeqprgggptsseqimktgalllqgfiqdragrmggeap
bcl-x                                                          msqSNRELVVDFLSYKLSQKGYSWsqfsdveenrteapegtesemetpsaingnpswhladspavngatghsssl
mcl-1                                              ...(+123 aa)eldgyepeplgkrpavlpllelvgesGnntstdgslpstpppaeeeedelyrqsleiisrylreqatgakdtk
A1                                                                                                                  maeselmhihslaehylqyvlq
bhrf                                                                                                    maystreillalcirdsrvhgngtlhpvlelaar
LMW5-HL                                                                                                                megeeliyhniineilvgy
ced9      mtrctadnsltnpayrrrtmatgemkeflgikgteptdfginsdaqdlpspsrqastrrmsigesidgkindweeprlDIEGFVVDYFTHRIRQNGMEWfgapg

cdn1      lplqpsstmgQVGRQLAIIGDDINRRYDSEFQTMLQHLQPTAENAYEYFTKIATSLFESGI-NWGRVVALLGFGYRLALHVYQHGLTGFLGQVTRFVVDFMLHH
cdn2      lplqpsstmgQVGRQLAIIGDDINRRYDSEFQTMLQHLQPTAENAYEYFTKIASSLFESGI-NWGRVVALLGFSYRLALHIYQRGLTGFLGQVTRFVVDFMLHH
bcl2      agpalspvppVVHLTLRQAGDDFSRRYRRDFAEMSRQLHLtpftargRFATVVEELFRDGV-NWGRIVAFFEFGGVMCVESVNREMSPLVDNIALWMTEY-LNR
bax       elaldpvpqdastkklseclkrigdeldsnmelqrmiaavdtdsprevFFRVAADMFSDGNFNWGRVVALFYFASKLVLKALCTKVPELIRTIMGWTLDF-LRE
bcl-x     darevipma-AVKQALREAGDEFELRYRRAFSDLTSQLHITPGTAYQSFEQVVNELFRDGV-NWGRIVAFFSFGGALCVESVDKEMQVLVSRIAAWMATY-LND
mcl-1     pmgrsgatsrkalETLRRVGDGVQRNHETVFQGMLRKLDIKNEDDVKSLSRVMIHVFSDGVTNWGRIVTLISFGAFVAKHLKTINQESCIEPLAESITD-VLVR
A1        vpafesapsqacrvlqrvafsvqkeveknlksylddfhvesidtariiFNQVMEKEFEDGIINWGRIVTIFAFGGVLLKKLpqeqialdvcaykqvssfvaefi
bhrf      etplrlspedtvvlryhvlleeiiernsetftetwnrfithtehvdldfnsvfleifhD-LINWGRICGFIVFSARMAKYCKDANn-HLESTVITTAYNF-SEG
LMW5-HL   ikyymndihelspyqqqikkiltyydeclnkqvtitfsltnaqeiktQFTGVVTELFKrgdpslgralawmawcmhacrtlccnqstpyyvvdlsvrgmleaM-
ced9      lpcgvqpehemmrvmgtifekkhaenfetfceqLlavprisfslyqdvvrtvgnaqtdqcpMSYGRLIGLISFGGFVAAKMmesvelqgqvrnlfvytslfIKT

cdn1      CIAR--WIA-QR-GGWVAALNLGngpilnvlvvlgvvllgqfvvrrffks
cdn2      CIAR--WIA-QR-GGWVAALNLGngpilnvlvvlgvvllgqfvvrrffks
bcl2      HLHT--WI--QDNGGWDAFVELYgpsmrplfdfswlslktllslalvgacitlgaylghk
bax       RLLG--WI--QDQGGWDGLLSYFgtptwqtvtifvagvltasltiwkkmg
bcl-x     HLEP--WI--QENGGWDTFVELYgnnaaaesrkgqerfnrwfltgmtvagvvllgslfsrk
mcl-1     TKRD--WLVKQ--RGWDGFVEFFhvedleggirnvllafagvagvgaglaylir
A1        MNNTGEWI-RQ-NGGWEdgfikkfepksgwltflqmtgqiwemlfllk
bhrf      -LDG--WIHQQ--GGWStliednipgsrrfswtlflagltlsllvicsylfisrgrh
LMW5-HL   KHNLLPWMISH--GGQEEFLAFslhsqiysvifnikyflskfcnhhflrscvqllrkcnli
ced9      -RIRNNWKE-H-NRSWDDFMTLgkqmkedyeraeaekvgrrkqnrrwsmigagvtagaigivgvvvcgrmmfslk
```

SEQUENCE IDENTITY:

cdn1/cdn2 = 97%

Sequence Range: 1 to 5408

Figure 7 cdn-3 enzyme annotated sequence

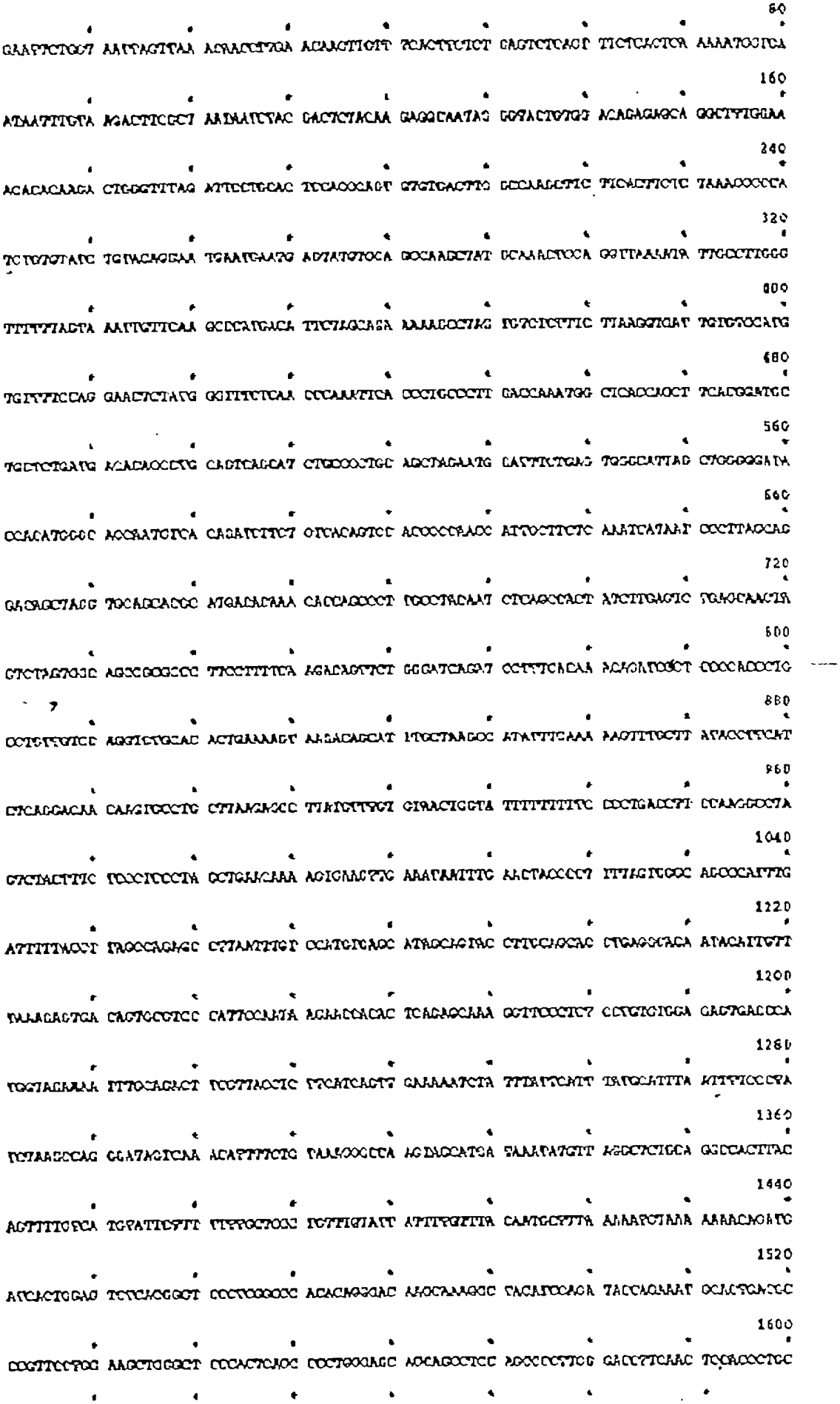


Figure 7 cont.

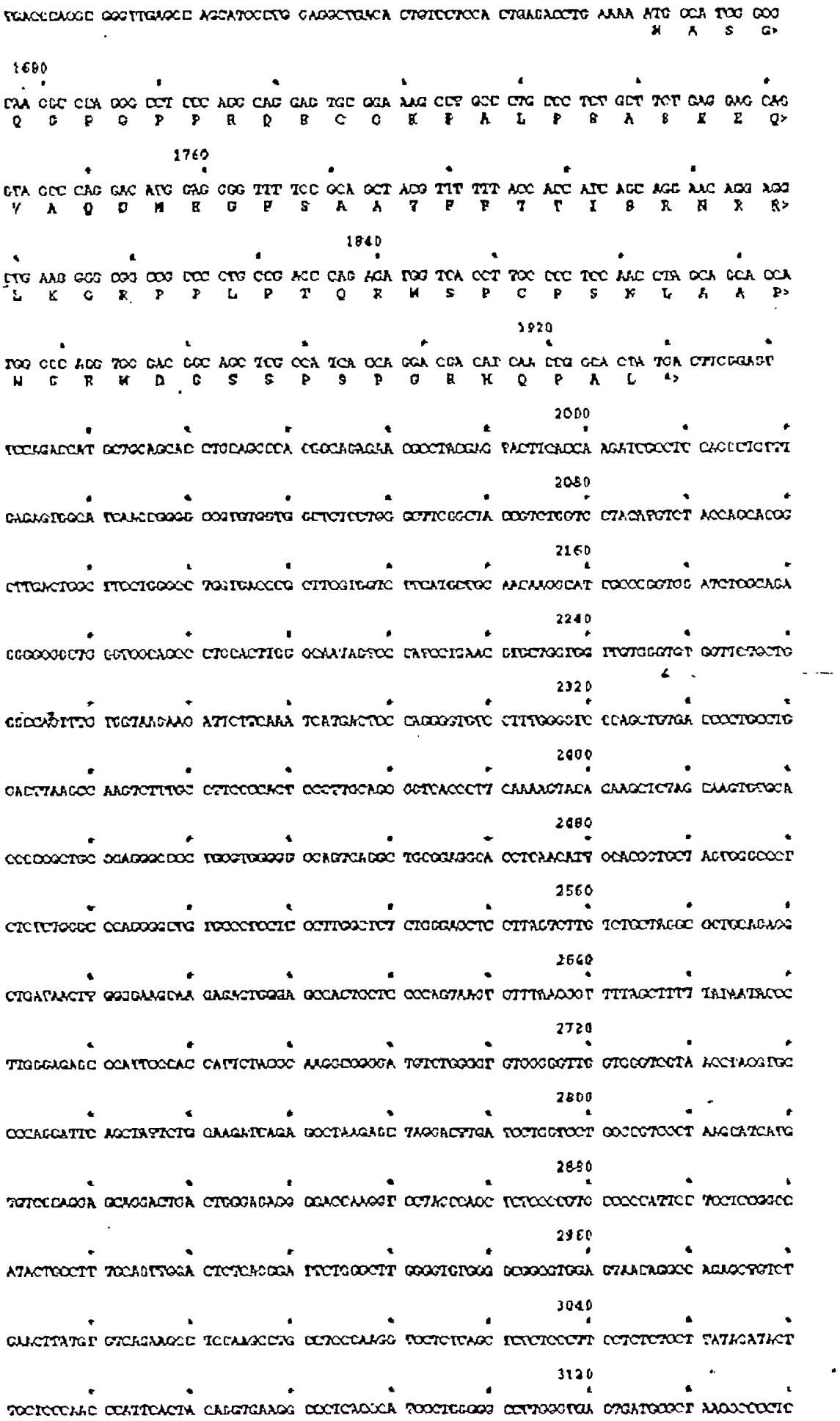


Figure 7 cont.

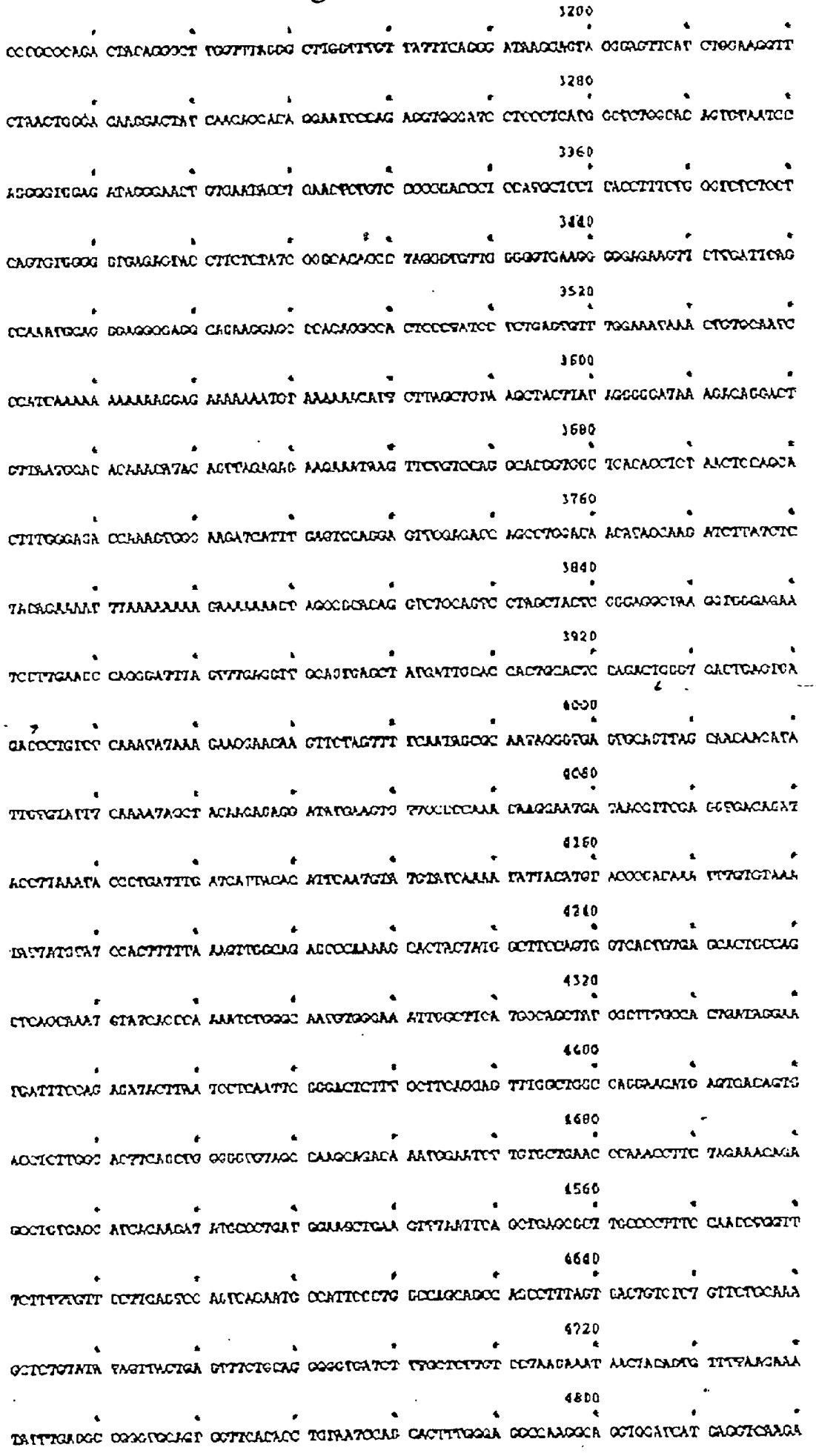


Figure 7 cont.

Figure 8

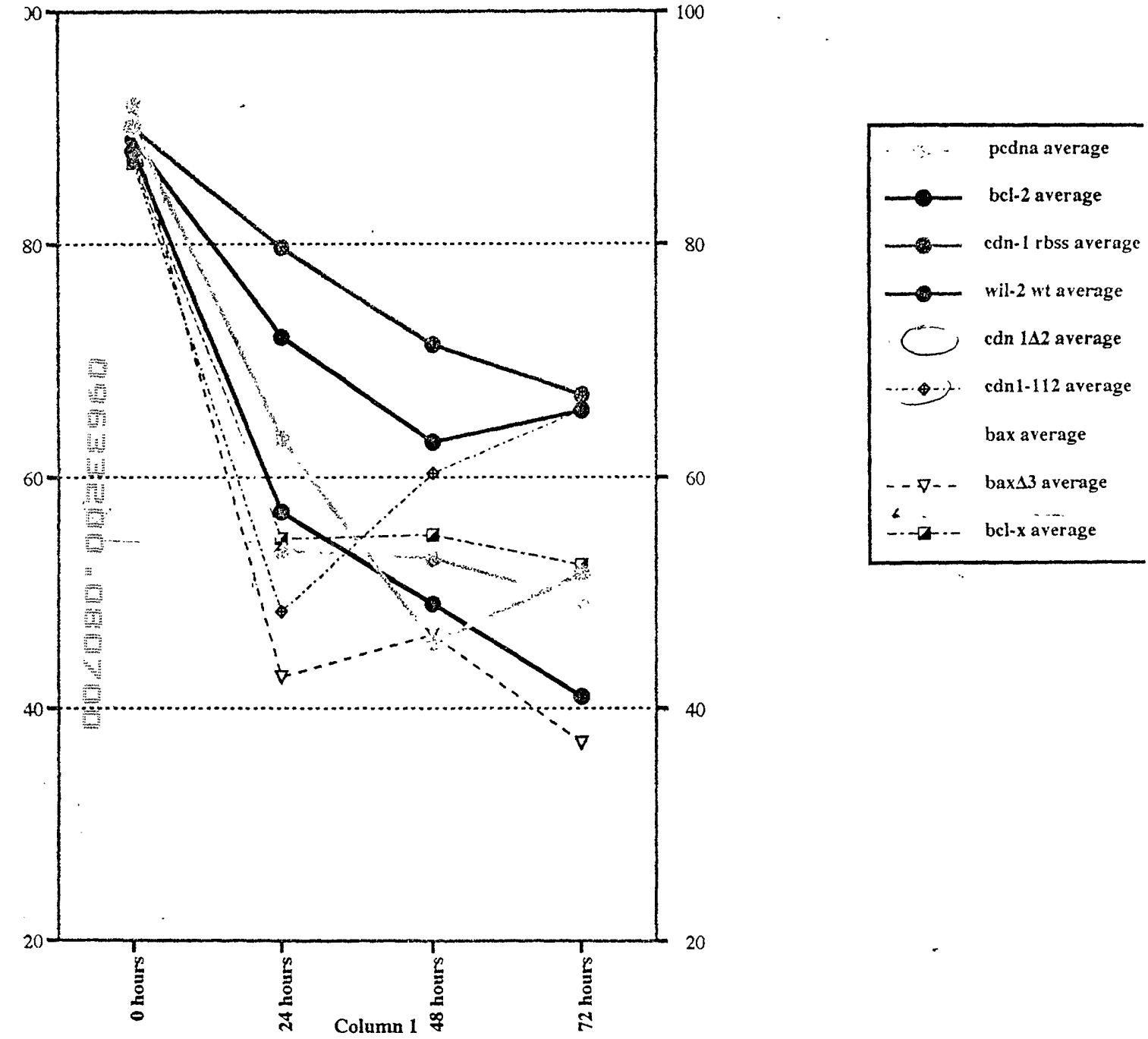

Wil-2 transformants 0.1% FBS
100
80
60
40
20
0 hours
24 hours
48 hours
72 hours
Column 1
pcdna average
bcl-2 average
cdn-1 rbss average
wil-2 wt average
cdn 1Δ2 average
cdn1-112 average
bax average
baxΔ3 average
bcl-x average

Figure 9

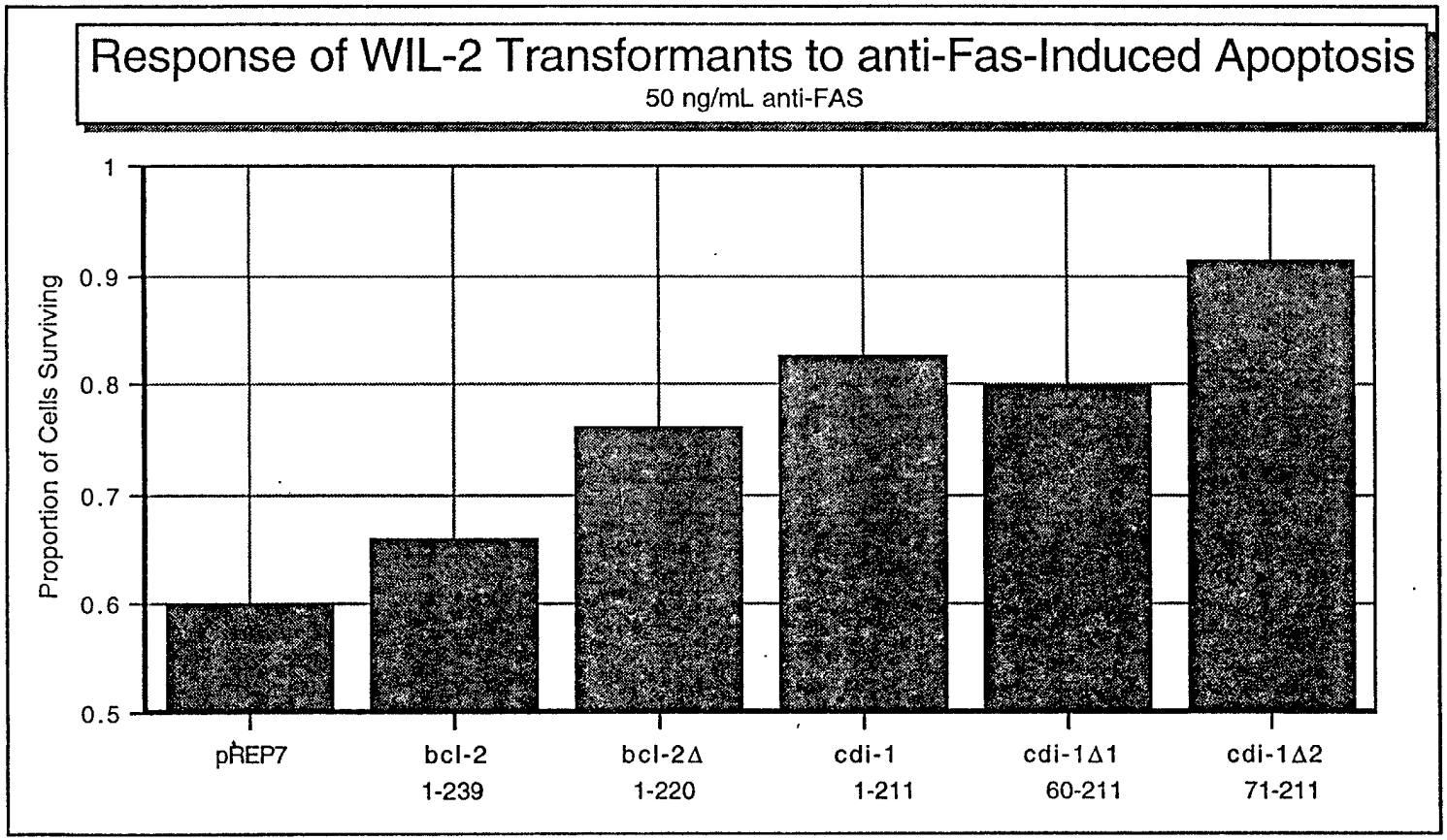


Figure 10

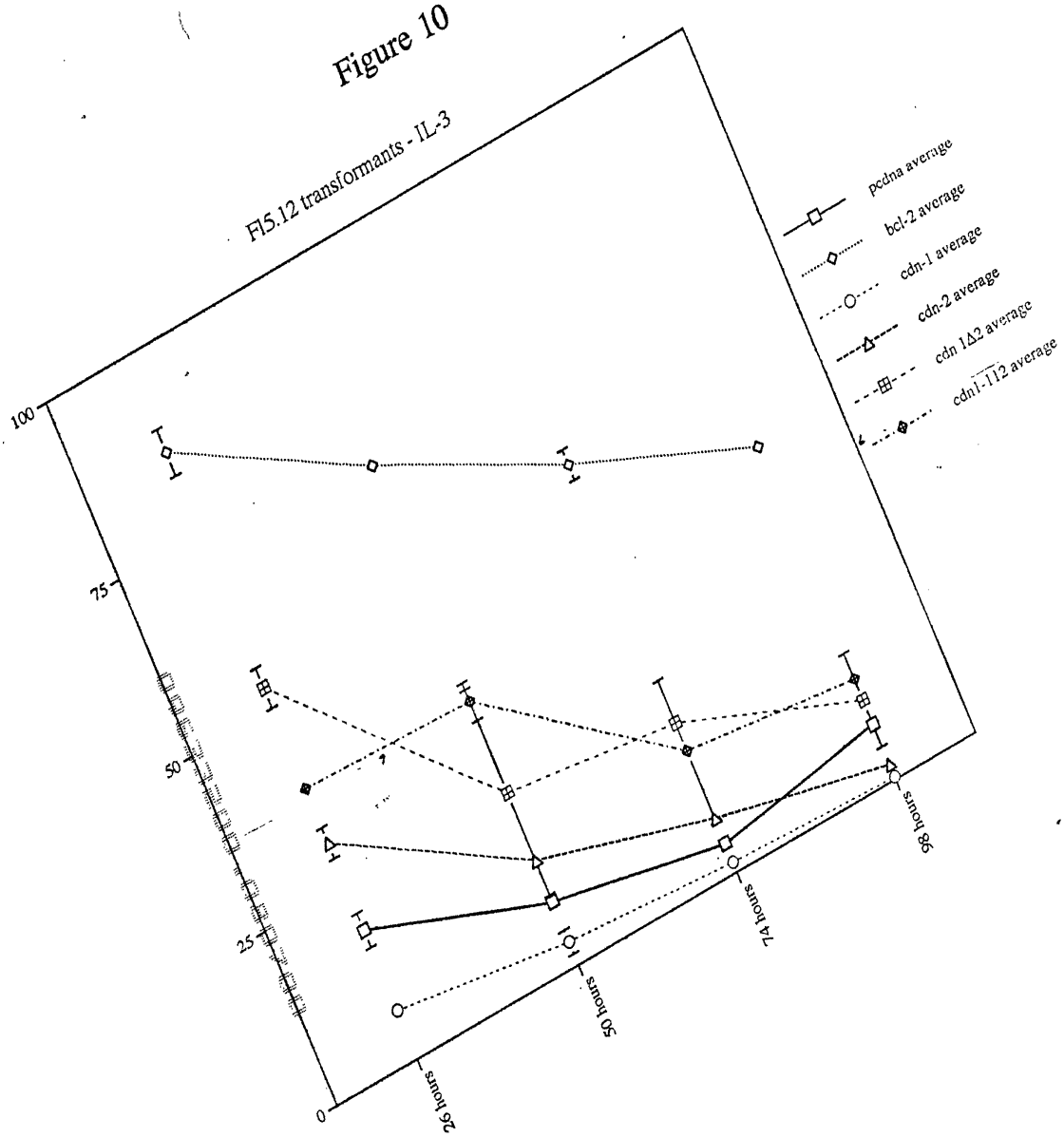
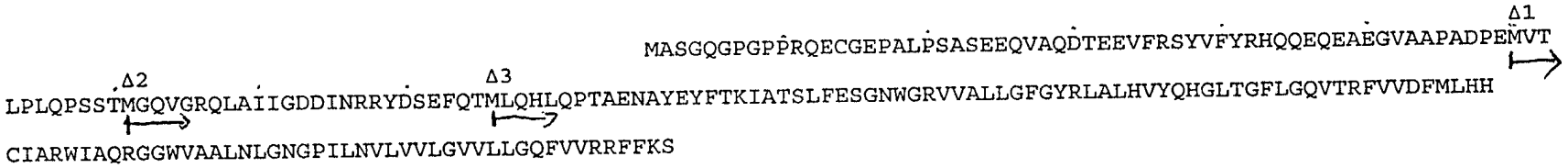

FI5.12 transformants - IL-3
100
75
50
25
0
26 hours
50 hours
74 hours
98 hours
pcdna average
bcl-2 average
cdn-1 average
cdn-2 average
cdn 1Δ2 average
cdn1-Δ12 average

Figure 11 N-terminal methionine residues of cdn-1 derivatives



COMBINED DECLARATION AND POWER OF ATTORNEY
FOR CONTINUATION-IN-PART APPLICATION

AS A BELOW-NAMED INVENTOR, I HEREBY DECLARE THAT:
My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if more than one name is listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: NOVEL APOPTOSIS-MODULATING PROTEINS, DNA ENCODING THE PROTEINS AND METHODS OF USE THEREOF, the specification of which

(check one) __ is attached hereto
_x_ was filed on October 7, 1994

as application serial no. 08/320,157 and was amended on (if applicable).

I HAVE REVIEWED AND UNDERSTAND THE CONTENTS OF THE ABOVE-IDENTIFIED SPECIFICATION, INCLUDING THE CLAIMS, AS AMENDED BY ANY AMENDMENT REFERRED TO ABOVE.

I acknowledge and understand that I am an individual who has a duty to disclose information which is material to the patentability of the claims of this application in accordance with Title 37, Code of Federal Regulations, §§ 1.56(a) and (b) which state:

> "(a) A patent by its very nature is affected with a public interest. The public interest is best served, and the most effective patent examination occurs when, at the time an application is being examined, the Office is aware of and evaluates the teachings of all information material to patentability. Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is cancelled or withdrawn from consideration, or the application becomes abandoned. Information material to the patentability of a claim that is cancelled or withdrawn from consideration need not be submitted if the information is not material to the patentability of any claim remaining under consideration in the application. There is no duty to submit information which is not material to the patentability of any existing claim. The duty to disclose all information known to be material to patentability is deemed to be satisfied if all information known to be material to patentability of any claim issued in a patent was cited by the Office or submitted to the Office in the manner prescribed by §§ 1.97(b)-(d) and 1.98. However, no patent will be granted on an application in connection with which fraud on the Office was practiced or

attempted or the duty of disclosure was violated through bad faith or intentional misconduct. The Office encourages applicants to carefully examine:

(1) prior art cited in search reports of a foreign patent office in a counterpart application, and

(2) the closest information over which individuals associated with the filing or prosecution of a patent application believe any pending claim patentably defines, to make sure that any material information contained therein is disclosed to the Office.

(b) Under this section, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and

(1) It establishes, by itself or in combination with other information, a prima facie case of unpatentability of a claim; or

(2) It refutes, or is inconsistent with, a position the applicant takes in:

(i) Opposing an argument of unpatentability relied on by the Office, or

(ii) Asserting an argument of patentability.

A prima facie case of unpatentability is established when the information compels a conclusion that a claim is unpatentable under the preponderance of evidence, burden-of-proof standard, giving each term in the claim its broadest reasonable construction consistent with the specification, and before any consideration is given to evidence which may be submitted in an attempt to establish a contrary conclusion of patentability."

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below, and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) and (b) set forth above which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No.: 08/160,067
Filing Date: November 30, 1993
Status (patented, pending, abandoned): pending

As to the subject matter of this application which is common to said earlier application, I do not know and do not believe that the same was ever known or used in the United States of America before my or our invention thereof or patented or described in any printed publication in any country before my or our invention thereof or more than one year prior to said earlier application, or in public use or on sale in the United States of America more than one year prior to said earlier application. Said common subject matter has not been patented or made the subject of an inventor's certificate issued before the date of said earlier application in any country foreign to the United States of America on an application filed by me or my legal representatives or assigns more than twelve months prior to said earlier

application; and the earliest application(s) for patent or inventor's certificate on said invention filed by me or my legal representatives or assigns in any country foreign to the United States of America is identified below, as well as all other such applications (if any) filed more than twelve months prior to the filing date of this application:

The priority of the earliest application(s) (if any) filed within a year prior to said pending prior application is hereby claimed under 35 U.S.C. § 119.

As to the subject matter of this application which is not common to said earlier application, I do not know and do not believe that the same was ever known or used in the United States of America before my or our invention thereof or patented or described in any printed publication in any country before my or our invention thereof or more than one year prior to the date of this application, or in public use or on sale in the United States of America more than one year prior to the date of this application. Said subject matter has not been patented or made the subject of an inventor's certificate issued in any country foreign to the United States of America on an application filed by me or my legal representatives or assigns more than twelve months prior to the date of this application; and the earliest application(s) for patent or inventor's certificate on said subject matter filed by me or my legal representatives or assigns in any country foreign to the United States of America is identified below, as well as all other such applications (if any) filed more than twelve months prior to the filing date of this application:

The priority of the earliest application(s) (if any) filed within a year to this application is hereby claimed under 35 U.S.C. § 119.

I hereby appoint the following attorneys and agents to prosecute that application and to transact all business in the Patent and Trademark Office connected therewith and to file, to prosecute and to transact all business in connection with all patent applications directed to the invention:

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Jackie N. Nakamura - Reg. No. 35,966
Freddie K. Park - Reg. No. 35,636
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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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